STUDIES ON THE GENETIC ENGINEERING OF HERBICIDE RESISTANCE INTO SOUTH AFRICAN TOBACCO CULTIVARS

BY

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CONTENTS

ABSTRACT

Tobacco is an important crop in South Africa. The genetic basis of tobacco breeding is very narrow and cultivars are closely related. The production of new tobacco hybrids with novel characteristics through classical breeding techniques is difficult. Genetic engineering could assist plant breeders to introduce new herbicide, disease and pest resistance traits into existing proven cultivars. Plant genetic engineering has not previously been applied to the improvement of South African commercial tobacco cultivars.

Agrobacterium-mediated leaf disc transformation was used to create transgenic tobacco plants from South African commercial tobacco cultivars TL33, J6 and 20/19. The cultivar samsun was also used to create transgenic plants. The *Agrobacterium tumefaciens* helper strain C58C1(pGV2260) containing the binary vector pJIT119 was used to carry out the transformation. As well as the leaf disc transformation method, other methods of obtaining transgenic tobacco plants were explored. These methods included the use of *Agrobacterium*-mediated transformation of tobacco cell cultures and direct DNA-mediated transformation of tobacco protoplasts.

The vector pJIT119 encodes the *uidA* gene for the ß-glucuronidase (GUS) enzyme, the *nptII* gene for neomycin phosphotransferase (NPTII) and the *sul* I gene for the dihydropteroate enzyme conferring asulam resistance. The presence and expression of these three foreign genes *uidA* , *nptII* and *sul* I from pJIT119 in transgenic tobacco

plants was confirmed by a variety of experimental approaches, including the culture of transgenic plants on medium containing kanamycin or asulam, the GUS histochemical assay, the neomycin phosphotransferase assay, DNA dot-blot analysis, *in situ* hybridization, computerized image analysis, polymerase chain reaction and progeny analysis. A detailed analysis of individual transgenic plants is necessary in order to select those plants which express the foreign genes maximally. Only these plants would be given to plant breeders for field trial assessment.

A high level of foreign gene inactivation was observed in transgenic tobacco plants obtained from the *Agrobacterium*-mediated leaf disc transformation method. Approximately 20% of the original transgenic plants were discarded as "escapes" as they contained a defective *nptII* gene. The remaining kanamycin resistant plants, however, had inactive copies of either the *sul* I or the *uidA* gene, or both. The use of *in situ* hybridization and the polymerase chain reaction (PCR) helped to explain the foreign gene inactivation. The lack of foreign gene expression in individual transgenic plants was not due to the physical loss of entire foreign genes, DNA methylation or the position effect. The lack of expression was due to possible T-DNA rearrangements or deletions which disabled certain genes carried on the T-DNA. Transcription and translation of these foreign genes occurred, but the final *uidA* and *sul* I gene products (ß-glucuronidase and dihydropteroate synthase, respectively) were possibly defective and did not confer GUS activity or asulam resistance on the transgenic plants.

The tissue specific activity of the *uidA* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter was studied. In the vegetative structures of transgenic tobacco plants, the *uidA* gene activity was located within the cells surrounding the vascular traces and within the glandular hairs. The effects of stress on 35S promoter activity was also investigated. Chemical and nutrient stess *in vitro* did not have a significant effect to decrease *uidA* gene expression under 35S promoter control. Foreign gene expression (*uidA*) under CaMV 35S promoter control may be enhanced by *in vitro* stress. Oxygen stress (anaerobic culture under waterlogged conditions) induced *uidA* expression in areas of the plant which usually did not show usual tissue specific patterns of *uidA* expression. The stage of differentiation in tissue culture when compared to the mature hardened off transgenic plant, also had an effect on the amount of *uidA* gene expression. Mature hardened off plants expressed less GUS activity than immature *in vitro* plants.

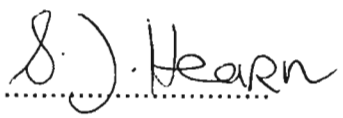
The tissue specific pattern of foreign gene expression under CaMV 35S promoter direction was conserved in the reproductive structures of transgenic tobacco plants. In floral organs, the pattern of *uidA* gene expression was essentially the same as that found in vegetative tissues. In all floral organs examined, *uidA* expression was found associated with the vascular system and within the glandular hairs. The *uidA* gene with a CaMV 35S promoter was not expressed in pollen.

Because of the ease of transformation of tobacco, it is possible that genes for

pharmaceutically valuable proteins and peptides could be expressed in tobacco, for agricultural scale fine chemical production("pharming"). This could be of economic advantage for the survival of tobacco as a commercial agricultural crop in the future when tobacco smoking is no longer popular.

DECLARATION

I hereby declare that this thesis, unless acknowledged to the contrary in the text, is the result of my own investigation under the supervision of Doctor William Cress, Department of Botany, University of Natal, Pietermaritzburg and co-supervision of Doctor Jocelyn Webster, Biotechnology Programme, Division of Food Science and Technology, CSIR, Pretoria.



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ABBREVIATIONS

Ap	ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
CsCl	cesium chloride
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
FDA	Food and Drug Administration
GMO	Genetically modified organism
GUS	ß-glucuronidase enzyme
kb	kilobase pairs
Kan	kanamycin
LB	Luria-Bertani broth
mRNA	messenger RNA
nptII	gene coding for neomycin phosphotransferase II
ng	nanogram
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pg	picogram
r	(superscript) resistance
RNA	ribonucleic acid
s	(superscript) sensitivity
SAGENE	South African Committee for Genetic Experimentation
SDS	sodium dodecyl sulfate
sul I	gene encoding dihydropteroate synthase
TBE	tris-borate EDTA buffer
T-DNA	transfer DNA of *Agrobacterium tumefaciens*

TE	tris EDTA buffer
TEA	triethanolamine buffer
Tris	tris(hydroxymethyl)aminomethane
U	units of enzyme activity
uidA	gene encoding the enzyme ß-glucuronidase (GUS)
USA	United States of America
USDA	United States Department of Agriculture
UV	ultraviolet (light)
V	volts
w/v	weight/volume
α	alpha
ß	beta
λ	lamda
μ	micro

Chapter 1

General introduction

Chapter 1

GENERAL INTRODUCTION

1.0 Introduction to plant genetic engineering.

Plant genetic engineering is a complex multidisciplinary approach to plant manipulation and plant improvement. It involves the introduction of modified heterologous DNA into plant cells. These plant cells must survive the manipulation process and regenerate into fertile plants expressing the new DNA (Cocking, 1990; Lindsey, 1992). A system based on the natural gene transfer properties of a soil-borne plant pathogen, *Agrobacterium tumefaciens*, initiated the age of plant genetic engineering in the 1980's. As well as *Agrobacterium*-mediated transformation, many different gene transfer systems have been developed to introduce new genes into plants. Chemical techniques (calcium-DNA co-precipitation, liposome fusion and polyethylene glycol treatment), electrical techniques (electroporation), microinjection of DNA into intact plant cells, sonication (Joersbo and Brunstedt, 1992), vacuum infiltration (Bechtold *et al.*, 1993; Bouchez *et al.*, 1993) and DNA-coated microprojectile bombardment of cells of intact plant tissues are also employed to yield fertile transgenic plants (Lindsey, 1992). Ongoing developments in recombinant DNA technology and plant tissue culture have also contributed to the current success of plant genetic engineering.

Plant genetic engineering, part of the larger arena of plant biotechnology, is beginning to make an impact on world agriculture. Plant genetic engineering enables crop plants to be improved in an efficient and specific way, allowing new cultivars to be developed rapidly. It also makes possible novel non-food uses for agricultural crops (Sijmons *et al.*, 1990; McCormick, 1992; Pen *et al.*, 1992; Visser and Jacobsen, 1993). As well as these aspects, the science of plant genetic engineering is also enabling rapid advances to be made in mankind's knowledge of the molecular biology of plants (Nacy *et al.*, 1988).

1.1 Commercialization of plant biotechnology, including plant genetic engineering.

The common goal of modern biotechnology companies is to use biological processes and systems for the production of commercial goods or services. Biotechnology business interests presently include human health care, agricultural productivity, animal health, food safety and nutrition, and chemical and environmental improvement (Burrill and Lee, 1992). At present there are about 150 research organisations and 500 companies in existence around the world which have at least some interest in plant biotechnology research. These organizations and companies have made a substantial financial commitment in plant biotechnology and hope ultimately to have a good return from this investment (Vasil, 1990; Burrill and Lee, 1992). The rewards from biotechnology will be realized only in the long term and for this reason, the development of plant biotechnology is primarily funded by large multinational companies. These organizations have the level of capital required to fund long term research (Hansen *et al*., 1986; Beer, 1989). It is predicted that the value of the worldwide biotechnology market will rise to $120 billion (£180 million) by the turn of the century (Chen, 1994). The long term scientific and commercial implications of biotechnology are thought to be so enormous that they are beyond current financial analysis (Bock, 1993).

Agriculture is the world's largest industry (McCormick, 1992). This, coupled with the urgent need to produce better food more efficiently, and the possibility of growing lucrative non-food crops, indicates that international agriculture represents substantial business interests (Leemans, 1993; Ernst and Young's Seventh Annual Report on the Biotech Industry, 1992). The availability of useful genes and efficient transformation systems for crop species is therefore of intense commercial interest to the major agrochemical companies of the world (Gasser and Fraley, 1989; Fraley, 1992).

As well as technical barriers to successful production of transgenic products, certain non-technical barriers exist which could delay the commercialization of plant biotechnology (Fraley, 1992). The existence of strict regulatory requirements in certain countries, the lack of international [illegible]

genetically engineered organisms, the need for proprietary protection for novel germplasm and technology, and negative public perceptions on the desirability of transgenic products need to be addressed in order to clear the way for a co-ordinated world effort to commercialize biotechnology (Fraley, 1992). Legislation is being reviewed on a constant basis in the United States of America (USA) and in Europe, and as time proceeds, many of the problems associated with commercialization the products of biotechnology will be overcome.

1.2 The role of plant genetic engineering in agricultural crop improvement.

The role of plant genetic engineering in agricultural crop improvement will be to augment classical breeding methods, rather than replace them (Jones, 1992; Lindsey, 1992; Leemans, 1993). Classical plant breeding relies on sophisticated applied Mendelian genetics to produce new varieties of crop plants to meet farmer and consumer demands. Classical breeding has been successful over the last 50 years in developing high yielding, disease resistant varieties of crop plants (Watson *et al.*, 1983). However, plant breeding is a slow process and is limited to sexually compatible species. Plant genetic engineering can overcome these limitations. Through the use of genetic engineering, plant breeders will be able to introduce single specific genes into already highly developed and characterized varieties of crop plants. Future transgene technology will also allow for multiple independent transgenes to be sequentially added to a cultivar as they become available, to continually upgrade a cultivar of proven value (Yoder and Goldbrough, 1994). Genetic engineering can be a rapid method of crop improvement, but only once the tissue culture and transformation procedures have been optimized (Jones, 1992). The process of identifying and locating new genes to introduce into plants is also not simple and has required a considerable research effort and funding over the last decade.

Despite the technical difficulties, the rapid rate at which the applications of this research have unfolded has been exceptional. In the USA, for example, field trials of transgenic crop plants are now a routine event. This is a considerable achievement considering that the first successful genetic engineering

Estrella *et al.*, 1983). At present, the largest number of transgenic field trials are conducted in the USA.

Since the United States Department of Agriculture (USDA) started regulating field trials involving transgenic crop plants in 1986, more than 370 permits in 35 states in the USA have been issued (Leemans, 1993). In 1992 alone, field test applications for transgenic crops included 41 applications for maize, 32 applications for soybeans, 26 applications for potatoes, 18 applications for tomatoes and 13 applications for cotton were made. Agronomically valuable traits which have been transformed into plants for field testing in the USA include herbicide tolerance, virus resistance and insect resistance. Transgenic plants with increased starch levels (Kawasaki *et al.*, 1993) or additional seed storage proteins (DeLisle and Crouch, 1989; Leisy *et al.*, 1989; Altenbach *et al.*, 1992) and ripening characteristics modified through genetic engineering (Kareiva, 1993) are also undergoing field testing in the USA.

The transgenic field trials performed in the USA up to the present have proved successful. Biotechnology companies conducting these trials have now sought the deregulation of their transgenic genotypes to enable these recombinant plant varieties to be treated as conventional crops. This is a prerequisite for the commercialization of transgenic plants to obtain a return on investment (Agrow, number 172, November 1992, page 18). The commercialisation of transgenic crops in the USA was also advanced in 1992 following the issue of a policy statement by the Food and Drug Administration (FDA). The statement made was to the effect that transgenic food crops would be regulated no differently from conventional crops unless the genetic modification raised particular safety questions (Genetic Technology News, June 1992, page 3). Later that year, the USDA proposed replacing its current permit-based field trial system for transgenic crops with a simple notification procedure. This change would accelerate field testing and the subsequent commercialization of transgenic crop varieties (Genetic Technology News, January 1992, page 2; Agrow, number 172, November 1992, page 18).

In addition to improving agricultural crops for food production, plant genetic engineering also has the potential to extend the commercial use of [illegible]

agriculture based on meeting the basic food needs of Europe (Second European Symposium on Industrial Crops and Products, final notice, 1993). In such a scenario, substantial arable land in Europe would be under utilized. To extend the role of agriculture in Europe and make full use of agricultural land, the modification of plants through plant genetic engineering could result in new industrial and non-food uses for crops (Swain, 1991). The next generation of genetically engineered plant products could therefore be designed to serve the needs of the food processing, speciality chemical and pharmaceutical industries, rather than be consumed for food (Fraley, 1992). One could envisage food shortages in Europe associated with high food prices, as farmers substitute lucrative industrial "cash crop" agriculture for food production. In Africa, the demand will, for some time to come, still be for more efficient and sustainable food production, and plant genetic engineering will be required to contribute disease and pest resistant crop plants for third world agricultural production (Robertson and Sakina, 1989).

Whether biotechnology will bring opportunities for agriculture in the Third World remains to be seen. The focus of First World biotechnology has been to develop commercially viable, market-driven plant products (eg. tomatoes with an extended shelf life) rather than develop products to enhance sustainable, small-farmer agriculture. Biotechnology may even pose a threat to Third World farmers through the loss of indigenous agricultural germplasm. Many of the crop varieties in use in the Third World have been selected and domesticated over hundreds of years and these could be lost in favour of genetically engineered varieties supplied by First World biotechnology firms seeking to penetrate new markets (Shand, 1993). Third World economies are also under jeopardy from First World biotechnology. In Africa alone, $10 billion in exports are vulnerable to industry-induced changes in raw material prices and requirements (Shand, 1993). Biosynthesis of high-value ingredients like vanilla, pyrethrum and rubber, will untimately transfer production out of farmers' fields into the industrial biotreactors of Western countries (Cohen, 1989; Goldstein, 1991; Shand, 1993).

1.3 Present and future areas of crop improvement using plant genetic engineering.

The early research into plant biotechnology, funded by the large international seed and agrichemical companies, initially concentrated on traits which reflect the traditional role of these industries in agriculture, namely assisting farmers to combat pests, diseases and weeds (Fraley, 1992 and Leemans, 1993). Modern crops, by virtue of their monoculture cultivation, are highly susceptible to attack by insect pests, viral, bacterial and fungal diseases. Competition from weeds also results in heavy yield and quality losses (Llewellyn *et al.*, 1990; Leroux *et al.*, 1990). Usually chemical products (i.e. biocides, herbicides) are used to check the spread of these infestations, but the heavy use of chemicals in agricultural in becoming increasingly unpopular. Plant genetic engineering can provide novel strategies that do not require the application of agricultural chemicals to protect crops from pests and diseases.

To minimize the need for multiple applications of pesticides, transgenic crops containing genes to protect the plants against lepidopteran insect pests can be produced (Leemans *et al.*, 1990; McDonald, 1992). Specific bacterial genes coding for a variety of delta endotoxin proteins have been isolated from several strains of the entomocidal bacterium *Bacillus thuringiensis*. These proteins are toxic to lepidopteran insects. The genes confer resistance to these insect pests when transferred into plants (Fischoff *et al.*, 1987; Meeusen and Warren, 1989; Delannay *et al.*, 1989). Caterpillar-resistant transgenic crop plants are already undergoing field trials in the USA (Meeusen and Warren, 1989). Additional *B.thuringiensis* strains with activity against dipteran and coleopteran insect pests have also been isolated and could also find use in protecting plants from damage by these insects (Hernstadt *et al.*, 1986; Delannay *et al.*, 1989).

Another class of insecticidal proteins known as proteinase inhibitors can also be exploited through the use of plant genetic engineering to develop transgenic plants which are protected from insect damage (Brunke and Meeusen, 1991). The cowpea (*Vigna unguiculata* L. (Walp.)) trypsin inhibitor protein, for example, disrupts the gut

gene for this trypsin inhibitor has been cloned. When transferred into plants using plant genetic engineering techniques, the trypsin inhibitor gene confers some measure of field resistance to insect pests on the plants (Gould, 1988; Brunke and Meeusen, 1991).

Genetic engineering can provide very specific control systems for virus infections. Plants transformed with a viral gene encoding a viral coat protein are resistant to infection by that virus or by closely related viruses. Although the exact mechanism for this resistance is unknown, this phenomenon has been observed for the major crop infecting viruses such as potato virus X and Y, tobacco mosaic virus, alfalfa mosaic virus, soybean mosaic virus and cucumber mosaic virus (Hill *et al.*, 1991; Jones, 1992; Lindsey, 1992). The inclusion of viral coat protein genes does not affect vegetative traits of the transgenic plant or fruit production (Nelson *et al.*, 1988). Antisense technology can also be used to genetically engineer virus resistance into plants (Bejarano and Lichtenstein, 1992).

There is also a role for plant biotechnology to devise novel strategies to obtain plants resistant to fungal diseases (Cornelissen and Melchers, 1993). Plants use various defense mechanisms to protect themselves against infection by fungal pathogens. Plants can produce chitinases and ß-1,3-glucanases and other products which attack fungal cells (Cornelissen and Melchers, 1993). There is great interest in cloning the genes for these enzymes and transforming them back into plants to achieve increased plant resistance to fungal attack (Dixon and Harrison, 1990; De La Fuente-Martinez *et al.*, 1992). By cloning specific antibody genes and transferring them into plants, it is possible that disease and nematode resistance could be obtained as well (Hiatt *et al.*, 1989; Hiatt, 1990; Tavladoraki *et al.*, 1993).

Herbicide resistant crop plants will probably be one of the first genetically engineered products containing traits of direct benefit to farmers to reach commercialization (LeBaron, 1987; Schulz *et al.*, 1990). The use of herbicides is a well-established necessity in modern agricultural practice. However, in recent years, there has been an increased demand for less toxic chemical herbicides. Several environmentally safe herbicides have been developed, but these are

crop plants allows the wider use of these herbicides (LeBaron, 1987; Schulz *et al.*, 1990).

There are three different genetic engineering approaches which can be used to obtain herbicide resistant crop plants. The first method of achieving herbicide resistance is to genetically engineer the overproduction of a herbicide-sensitive biochemical "target" within the cell (Stalker, 1988). The second approach is by genetically engineering a structural alteration of the herbicide "target" in the cell, resulting in reduced herbicide affinity (Stalker, 1988). Thirdly, the herbicide can be detoxified before it reaches the biochemical "target" inside the plant cell. The "targets" within the plant cells are enzymes, electron binding proteins or pathways. Resistance obtained by the first two mechanisms has been developed for the herbicides glyphosate, atrazine, the sulfonylureas and phosphinothricin (Stalker, 1988). The third mechanism has been achieved by producing transgenic plants expressing a bacterial gene encoding an enzyme that breaks down 2,4-dichlorophenoxyacetic acid (2,4-D) (Jones, 1992).

Many other new characteristics would have considerable commercial value to farmers if they could be introduced into plants through plant genetic engineering. These characterists include drought resistance, tolerance to flooding and poor soil aeration, tolerance to salt or toxic metals, the ability of plants to absorb and utilize fertilizers more efficiently, the abililty of non-leguminous crop plants to fix nitrogen (Cocking, 1990), an increase in the photosynthetic efficiency of crop plants, the development of crops with a shorter growing season, and the development of crops with an increased crop yield. However, these are all complex characteristics involving more than one gene and currently are difficult to manipulate directly using plant genetic engineering techniques. However, this type of crop improvement is being contemplated, but involves long term research (LeBaron, 1987).

More recently, attributes relating, not to the requirements of farmers, but to the needs of the consumer and the food industry have been investigated using plant genetic engineering techniques. The traits which could be altered to suit market trends include fruit ripening characteristics, nutritional composition, palatability and texture (Jones, 1992). In fact, the first transgenic food product to come on the

market in the USA will not be a herbicide, pest or disease resistant crop plant. It will, instead, be a transgenic tomato developed by Calgene. This product, the Flavr Savr tomato, has been modified to conform with the consumers requirement for a naturally ripened tomato with an extended shelf life (Van Brunt, 1992). The ripening of tomato can be delayed through the use of antisense technology to eliminate a specific enzyme involved in ripening, polygalacturonase (PG) (Fray and Grierson, 1993).

As well as producing new plants for agriculture, genetic engineering of crop plants could have impact on certain industries which use plant material as a major input. Some examples are outlined below. In the brewing industry, malting could be enhanced through the use of a transgenic barley, genetically engineered to contain improved ß-glucanases (Von Wettstein, 1989). The subunit genes from the wheat seed storage protein glutenin have been cloned, and the use of these genes in improving the bread-making properties of wheat is being investigated (Macritchie, 1992). Soy protein is used extensively in foods as an emulsifier, but its properties become limited at low pH when the large globulins become insoluble. Modification of the composition of these globulins by genetic engineering would result in improvements in the properties of these proteins at low pH (Jones, 1992). The genetic modification of plants for altered starch content and composition is also being considered to create novel starches with industrial uses (Visser and Jacobsen, 1993).

A genetic engineering approach is also being used to modify perishable produce so that they can be stored at freezing temperatures without changes in texture and flavour. Fish antifreeze protein genes have been expressed in plant cells and give some protection against ice damage (Georges *et al.*, 1990). This new characteristic would benefit food producers, wholesalers, retailers and the consumer.

The use of genetic engineering to modify the nutritional composition of the plant product is also being investigated. One area where this technology will have an impact is in the production of improved animal feedstuffs. Animal feeds based on maize or on the oil-cake residue of soybean or sunflower seeds, are deficient in

rich protein from brazil nut has been inserted into a variety of plants which subsequently produced seeds with significantly increased methionine contents (Altenbach *et al.*, 1992). Through genetic engineering animal feed based on the oil cake residues could be protein enriched to make it a more balanced food for livestock. The genes for the seed storage proteins, zein, ß-conglycinin, and phaseolin, have been transferred into oilseed plants for this purpose (Beachy *et al.*, 1985; Matzke *et al.*, 1990).

The modification of the oil composition of oilseed crops is also target of genetic engineering. It may soon be possile to cultivate transgenic oilseed crops capable of synthesizing a large spectrum of novel, industrially useful oils (Derksen *et al.*, 1993; Murphy, 1993; Thierfelder *et al.*, 1993). Also, plants producing edible oils or oils which are used as industrial raw material could be modified to improve their agronomic performance and to alter the type and/or quality of oil produced (Murphy, 1993; Thierfelder *et al.*, 1993). Oil type and quality can be manipulated through changing the degree of saturation of the fatty acids, or by modifying the length of fatty acid chains (Knauf, 1987; Battey *et al.*, 1989 ; Hildebrand, 1992; Rattray *et al.*, 1992; Murphy, 1993; Thierfelder *et al.*, 1993).

An extremely promising area of crop development facilitated by genetic engineering is the expression of commercially interesting heterologous proteins and peptides in plants (Hiatt *et al.*, 1989; Pen *et al.*, 1992). This development could lead to the production of pharmaceutical proteins and other valuable chemicals on an agricultural scale ("pharming") using traditional crops modified through genetic engineering. The production of antibodies, human serum albumin (Sijmons *et al.*, 1990), encephalins, interferon, insulin, neuropeptides, blood factors, growth factors and industrial enzymes (Pen *et al.*, 1992; Pen *et al.*, 1993) could be achieved in a variety of transgenic crops. This would combine the advantages of high production volumes and low operating costs using established agricultural systems (Krebbs and Vandekerckhove, 1990; Vasil, 1990; Swain, 1991; Jones, 1992; Turner, 1993).

Finally, in the future it will probably be possible to alter the taste, smell or colour of a plant product through genetic engineering (Jones, 1992). Altering the flavour of

through the use the gene which codes for the production of the protein thaumatin, which is the sweetest substance known (Ledeboer *et al.*, 1984; Witty, 1990). The thaumatin gene has been expressed in potato plants and transgenic potatoes could be grown agriculturally as a reliable commercial source of this sweet protein. Also, plants genetically engineered to contain thaumatin actually taste sweeter, and might find a market as flavour enhanced products (Jones, 1992; Kurihara and Nirasawa, 1994). By genetically engineering a bacterial fructosyltransferase gene into plants, the taste of the plant could be altered. Also, these plants could become an industrial source of fructose (Ebskamp *et al.*, 1994). Research is also being carried out to control colour in plant tissues (Jones, 1992). Currently, this work is limited to attempting to change the flower pigmentation of horticulturally important plants (Elomaa *et al*, 1993), but it might prove feasible in the future to use genetic engineering to control pigment production and accumulation in fruits and vegetables (Jones, 1992).

The genetic engineering of plants has not come about solely because of its application in directly improving crop plants. Plant genetic engineering techniques are also leading to new insights into the way plants function at the molecular level. As knowledge of the molecular processes of plants increases, further innovation in the utilization of plants could result in the future (Nacy *et al.*, 1988).

These examples illustrate the many diverse ways in which genetic engineering can be employed to improve crop plants or extend the manner in which plants are utilized. Because of recent innovations in plant genetic engineering and plant biotechnology, plant biology is set to enter a phase when both basic research and commercial applications will be limited only be research creativity and by research funding (Fraley, 1992).

1.4 Genetic engineering of crop plants for herbicide resistance.

Of the more than 347 permits granted by the United States Department of Agriculture (USDA) for field testing genetically modified plants between 1987 and early 1993, about one third were for transgenic herbicide resistance (Hoyle, 1993). In modern agricultural practice, the use of herbicides is a well-established

However, in recent years, the demand for environmental safety has led to pressure being placed on agrichemical companies to develop safer herbicides. Herbicides are now required that are rapidly biodegradable, that do not accumulate in the environment and that are not toxic to non-target organisms (Schulz *et al.*, 1990). Herbicides which conform to these specifications are available, but, few of these new chemicals fulfill the farmers' requirement for a selective herbicide. A selective herbicide controls weed plants, but has little effect on crop plants. Herbicide resistance genes expressed in transgenic crop plants would protect them against such broad spectrum herbicides.

Another common problem experienced by farmers are the effects of herbicides left over in the fields. During crop rotation, the second crop to be planted may be sensitive to herbicides used with the initial crop and which have a residual effect. Again, transgenic crops expressing herbicide resistance genes could be grown successfully as a follow-up crop in this situation (Schulz *et al.*, 1990). Because of these applications, the development of specific herbicide resistance is the goal of many plant breeding, tissue culture and genetic engineering programmes (Schulz *et al.*, 1990).

The major plant biochemical processes affected by herbicides are photosynthesis, respiration, nitrogen metabolism (including amino acid, protein and nucleic acid synthesis) and lipid metabolism (Akobundu, 1988). Herbicides for which resistance genes are available are mainly divided into those which inhibit photosynthesis and those which inhibit amino acid biosynthesis in plants (Botterman and Leemans, 1988; Oxtoby and Hughes, 1990).

Because photosynthesis is the main mechanism for energy capture and biomass production in plants, herbicides which interfere with photosynthesis are among the most important. The triazine herbicides (e.g. atrazine) and the herbicide bromoxynil both inhibit photosynthesis. Plants are killed because photosynthetic electron transport in photosystem II (PSII) is disrupted. Atrazine, for example, binds to the chloroplast thylakoid membrane, where it blocks electron transport at the second electron acceptor of PSII. This electron acceptor is the Q_B protein. The precursor of the Q_B gene is encoded by the *psbA* gene, which is a chloroplast gene (Schulz

et al., 1990). In plants, conventional breeding cannot make use of resistant mutants of chloroplast genes, and therefore the *psbA* gene is not useful in a conventional breeding programmes (Schulz *et al.*, 1990). Genetic engineering is a sound alternative in this situation.

In areas where atrazine has been extensively used, atrazine resistant weeds and bacteria have appeared due to mutations of the *psbA* gene. In the search for atrazine resistance genes to introduce into plants, mutant *psbA* genes were isolated from atrazine resistant weeds and bacteria from these areas. An interesting situation arose with the use of bacterial *psbA* genes. The gene product of *psbA* is required inside the chloroplast to be effective. The bacterial *psbA* genes did not have the required transport sequences. A chimaeric gene consisting of the coding sequence of a mutant *psbA* gene from an atrazine resistant weed, *Amaranthus hybridus*, was constructed with an added transit peptide sequence from RUBISCO (Ribulose 1,5 bisphosphate carboxylase/oxygenase) (Botterman and Leemans, 1984). This construct allowed the chimaeric *psbA* gene product to be transported from the cytoplasm into the chloroplast. Transgenic plants which had received the *psbA* gene with the transit peptide sequence showed increased tolerance to atrazine (Botterman and Leemans, 1984).

Bromoxynil (3,5-dibromo 4-hydroxybenzonitrile) is also a potent inhibitor of photosystem II in plants (Oxtoby and Hughes, 1990). A bacterial nitrilase gene, *bxn*, was isolated from a soil bacterium *Klebsiella ozaenae* (Stalker *et al.*, 1988). This gene encodes a specific bromoxynil-specific nitrilase which converts bromoxynil to a non-toxic primary metabolite 3,5-dibromo-4-hydroxybenzoic acid. Transgenic plants expressing the *bxn* gene under the control of promoter from the light-inducible tissue-specific ribulose bisphosphate small subunit gene are resistant to high doses of bromoxynil (Stalker *et al.*, 1988)

The second group of herbicides for which resistance genes have been isolated are those which inhibit certain amino acid synthesis pathways. These herbicides include glufosinate ammonium, phosphinothricin (PPT), the sulfonylureas and imadazolinones and glyphosate (Oxtoby and Hughes, 1990).

The herbicides glufosinate ammonium and L-phosphinothricin (PPT) are substances which irreversibly inhibit the amino acid biosynthetic enzyme, glutamine synthetase (GS) in bacteria and plants (Botterman and Leemans, 1988). L-phosphinothricin, a component of herbicidal tripeptide called "Bialophos", is produced industrially by culturing *Streptomyces hygroscopicus* in fermentors (Schulz *et al.*, 1990). "Bialophos" is marketed under the tradename "Herbiace" (Meiji Seika, Japan). Glufosinate ammonium is the ammonium salt of chemically synthesized PPT. The trade name of glufosinate ammonium is "Basta" (Hoechst). These herbicides have been very well studied in the context of finding resistance genes to introduce into plants (Thompson *et al.*, 1987). Two bacterial genes, the *pat* and the *bar* genes, have been isolated which detoxify PPT. The *bar* (basta resistance) gene was isolated from *Streptomyces hygroscopicus*, and the product of this gene converts PPT into an acetylated form which has no herbicide activity (Thompson *et al.*, 1987; Schulz *et al.*, 1990). From *Streptomyces viridochromogenes* Tü494 a phosphinothricin acetyl transferase gene (*pat*) was also discovered (Wohlleben *et al.*, 1988). Plants which have been genetically engineered to contain either of these two bacterial genes are resistant to high doses of commercial formulations of phosphinothricin and bialophos (Schulz *et al.*, 1990).

The sulfonylureas (e.g. chlorsulfuron) and a structurally unrelated group of herbicides, the imadazolinones, are both potent broad-spectrum herbicides which inhibit the branched-chain amino acid biosynthetic enzyme acetolactate synthase (ALS) (Mazur *et al.*, 1987; Haughn *et al.*, 1988). This inhibition leads to a block in the production of the amino acids leucine, valine and isoleucine. Mutant genes for ALS which are resistant to the sulfonylureas and imadazolinones have been found in bacteria, yeasts and plants (Oxtoby and Hughes, 1988). However, certain structural features of bacterial ALS genes make them unsuitable for transfer into plants. Mutant ALS genes from higher plants were sought, and genes isolated from mutant *Arabidopsis thaliana* plants resistant to the suflonylurea herbicides were used to create sulfonylurea-resistant transgenic plants (Mazur *et al.*, 1987; Oxtoby and Hughes, 1990).

Glyphosate (N-(phosphonomethyl) glycine) is the active ingredient of the herbicide with the trade name "Roundup" which is

Company, St. Louis, United States of America (Smith *et al.*, 1986). Glyphosate is currently the most extensively used non-selective, broad-spectrum herbicide in the world. The target enzyme of glyphosate is the shikimate pathway enzyme 5-enolpyruvyl shikimate-phosphate (EPSP) synthase. This pathway controls the biosynthesis of aromatic animo acids in plants and bacteria. Glyphosate tolerant *Petunia hybrida* cell lines were obtained under glyphosate selection, and were found to overproduce EPSP synthase as a result of gene amplification (Shah *et al.*, 1986). A cDNA library was made from these plants and a mutant EPSP gene isolated which conferred glyphosate resistance to transgenic plants when linked to the cauliflower mosaic virus 35S promoter. The gene for ESPS synthase isolated in this manner also contained a transit peptide sequence. The transit peptide facilitated transfer of the ESPS gene product into the chloroplast where it conferred full resistance to glyphosate (Shah *et al.*, 1986; Comai *et al.*, 1985).

A similar study was performed whereby glyphosate resistant transgenic tobacco plants were obtained through the expression of a cloned mutant bacterial EPSP synthase gene (*AroA*) isolated from *Salmonella typhimurium* (Comai *et al.*, 1985). The bacterial EPSP gene isolated by Comai *et al.* (1985) had no transit peptide sequence. This should have resulted in transgenic plants which were not fully resistant to glysphosate. Because there was no transit peptide, the bacterial EPSP synthase enzyme coud not be transported into the chloroplasts of the plant cells. But, interestingly, transgenic tobacco plants containing this cytoplasmically located EPSP gene product were still protected against glyphosate (Comai *et al.*, 1988).

Other potent herbicides inhibit neither photosynthesis nor the amino acid pathways. The compound 2,4-dichlorophenoxyacetic acid (2,4-D) is a plant growth regulator which mimics the action of auxins. It is also a very potent herbicide in high concentrations, and is commonly used to control broad leaved weeds among monocotyledonous crop plants (Streber *et al.*, 1989). Several genera of soil bacteria can break down 2,4-D by several multi-enzyme pathways (Akobundu, 1988). The most intensively studied organism which can break down 2,4-D is *Alcaligenes eutrophus* (Lyon *et al.*, 1989; Oxtoby and Hughes, 1990). This soil organism has a complex 2,4-D degrading pathway comprising six enzymes, which convert 2,4-D through a series of stages to

subsequently metabolized to non-toxic succinic acid. The genes for the six enzymes have been isolated (*tfdA* - *tfdF*). Fortunately, all six genes do not have to be transferred into plants in order to achieve 2,4-D resistance. The first enzyme in the pathway, 2,4-D monooxygenase, is encoded by *tfdA* and converts 2,4-D into 2,4-dichlorophenol (DCP). This product is less toxic to plants than 2,4-D itself. The *tfdA* gene has been transferred to plants and transgenic plants with this gene survive 48 times the usual field application rate of 2,4-D (Lyon *et al.*, 1989).

Asulam (methyl (4-aminobenzenesulfonyl) carbamate) is a sulfonamide herbicide of the N-phenylcarbamate group (Maybaker product information). Asulam is a substance which affects the folic acid biosynthesis pathway of plants and bacteria. It is commercially used to control grass weeds in sugarcane, for weed control in banana plantations, for control of dock and bracken in pastures, orchards and forestry and also for the control of wild oats (*Avena fatua*) in flax cultivation (Maybaker product information). A bacterial sulfonamide resistance gene, *sul* I, isolated from a bacterial R plasmid confers resistance to asulam when introduced into plants (Guerineau *et al.*, 1990). The *sul* I gene encodes a mutated dihydropteroate synthase (DHPS) enzyme which is insensitive to inhibition by sulfonamides. A RUBISCO transit peptide gene is also required for the effective action of DHPS in transgenic plant cells. The *sul* I gene is also useful as a selectable marker in plant genetic engineering (Yoder and Goldsbrough, 1994). Transgenic tobacco plants expressing the *sul* I gene were the object of this present study.

Many of the new-generation herbicides which have been developed and used in Europe and the USA are currently in use in South Africa. The herbicides used in South Africa for which resistance genes are available for plant genetic engineering include glyphosate (Monsanto), bromoxynil (marketed locally by Maybaker, Agrimark and Applied Agricultural Products), atrazine (marketed locally by Bayer, Staalchem, Shell and FBC), chlorsulfuron (marketed in South Africa by Bayer and FBC), 2,4-D (2,4 dichlorophenoxyacetic acid, marketed by FBC, Staalchem and Maybaker), asulam (Maybaker and Rhône-Poulenc), and glufosinate ammonium (marketed by Hoechst).

It may become increasingly necessary to produce transgenic crop plants containing resistance genes to these herbicides so that these herbicides can be utilized more fully in South African agriculture. It is important that the South African agricultural industry participates more fully in the biotechnological revolution which is occurring in the world today. It is to this end that this thesis is dedicated.

1.5 The molecular biology of *Agrobacterium tumefaciens*.

One of the foundations of modern plant genetic engineering was discovered more than eighty years ago when Smith and Townsend (1907) published an article identifying the causative agent of a neoplastic plant disease, crown gall, as a bacterium. This bacterium was *Agrobacterium tumefaciens*. Since then, a large number of scientists throughout the world have added to understanding how the crown gall organism is able to induce crown gall disease. This research led to the discovery that gene transfer occurred between bacterium and plant cell. Following this, it was realized that the gene transfer system of *Agrobacterium tumefaciens* could be exploited for the controlled genetic engineering of plants for commercial and scientific purposes (Hooykaas and Schilperoort, 1992).

Agrobacterium tumefaciens is placed in the bacterial family Rhizobiaceae, together with the closely related species, *Agrobacterium rhizogenes* and the related species *Rhizobium*, all of which carry out unusual plasmid-related manipulations of plants (Dellaporta and Pesano, 1981). Well over 61 plant families of gymnosperms and angiosperms are susceptible to *A. tumefaciens*, including 142 genera of dicotyledonous plants. With few exceptions, monocotyledonous plants are not susceptible to *Agrobacterium* infection. Only dicotyledonous plants can be reliably genetically transformed using an *Agrobacterium*-mediated gene transfer system (Conner and Meredith, 1989).

In addition to chromosomal DNA, all oncogenic strains of *Agrobacterium tumefaciens* possess a large (150 - 200 kb) tumour inducing (Ti) plasmid which carries the bulk of the genes necessary to induce a crown gall on susceptible plants (Kerr and Roberts, 1976; Ellis *et al.*, 1979; Holsters *et al.*, 1982; Hooykaas and Schilperoort, 1992). It is the Ti plasmid which has provided the key to plant

genetic engineering.

Agrobacterium tumefaciens strains and Ti plasmids can be classified according to the typical opines which are present in the tumours they induce. The commonest strains are therefore octopine, nopaline, leucinopine and L,L- succinamopine type strains (Hooykaas and Schilperoort, 1992). Octopine Ti plasmids primarily encode the synthesis of octopine, but also code for the synthesis of agropine, octopinic acid, lysopine and histopine. Nopaline Ti plasmids code for nopaline as well as ornaline and agropine synthesis. Agropine Ti plasmids are related to octopine plasmids, but code for agropine metabolism only (Godwin *et al.*, 1991). Octopine strains can utilize octopine but not nopaline, while nopaline strains catabolize nopaline, but not octopine. Of the three types of Ti plasmids, the nopaline and octopine Ti plasmids have been the most extensively studied (Armitage *et al.*, 1988).

The many different types of Ti plasmids found in strains of *Agrobacterium* have four regions of homology. Two regions of homology, the T-DNA (transferred DNA) region and the *vir* region are associated with tumour formation, whereas the other two Ti plasmid regions are involved with the conjugative transfer of the Ti plasmid and with the replicative maintenance of the plasmid within the bacterium (Hooykaas and Schilperoort, 1992). Typical octopine (pTiAch5) and nopaline (pTiC58) Ti plasmids are shown in Figure 1.1. The virulence (*vir*) and oncogenic (*onc*) determinants on the Ti plasmid are vital for the tumorigenicity of the bacterium. Chromosomally located, constitutively expressed genes (*chv* genes) are also important in determining the apparatus necessary for transfer of the T-DNA from *Agrobacterium* to the plant cells (Matthysse *et al.*, 1981; Matthysse, 1987; Cangelosi *et al.*, 1989). On the Ti plasmid, the 40 kb virulence region (*vir*) is composed of seven *vir* operons, four of which (*virA*, *virG*, *virB* and *virD*) are absolutely essential for the transfer process. The other three *vir* operons (*virC*, *virE* and *virF*) are necessary only on certain host species (Hooykaas *et al.*, 1984; Stachel *et al.*, 1986). With the exception of the *virA* and *virG* genes, the *vir* operons are not transcribed during normal vegetative growth (Melchers *et al.*, 1989; Hille *et al.*, 1986; Mozo *et al.*, 1992).

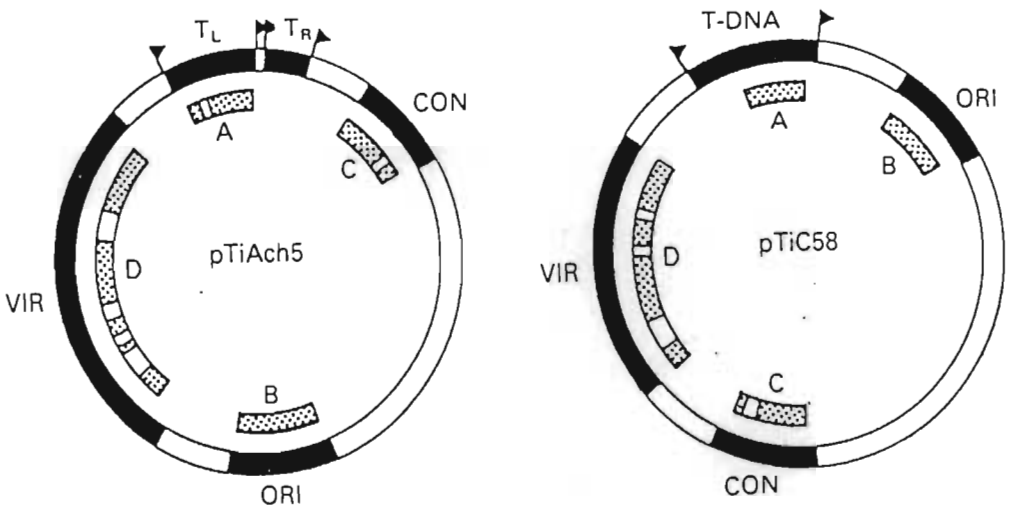

Fig. 1.1. General organization of an octopine-type (pTiAch5) and a nopaline-type (pTiC58) Ti plasmid. The relative positions and sizes of the major functional regions are shown. Shaded areas indicate the regions of extensive homology between the two plasmids. CON = regions encoding conjugation functions; ORI = region encoding replication functions and origin of replication; VIR = virulence region; T-DNA = region containing T (Transferred)-DNA; T_L, T_R = Left and right border

The T-DNA regions are flanked by 25 base pair direct repeats and the endpoints of T-DNA integration within the plant genome are found close to these sequences. The removal of the right border of a nopaline-type Ti plasmid abolishes tumour formation, since T-strand formation during T-DNA transfer begins at this border (Hooykaas and Schilperoort, 1992). T-strand formation is not initiated from the left border. The left border areas are much less efficient in acting as starting sites for DNA synthesis during T-DNA transfer than right borders because of the absence of an enhancer next to the right border sequence. This T-DNA transfer enhancer, also called "overdrive", strongly enhances T-strand formation (Peralta *et al.*, 1986; Mozo *et al.*, 1992).

During tumour formation, a defined sequence of the Ti plasmid, the T-DNA, is transferred from *Agrobacterium* to the plant cell and integrated into the plant nuclear genome. The T-DNA does not integrate at specific positions in the nuclear genome, but has a random pattern of insertion (Offringa *et al.*, 1990). The T-DNA is stable within the plant genome, and no major rearrangements of the sequences take place after T-DNA integration and during the establishment of the tumour. The T-DNA can be inserted more than once in the same plant genome during a transformation event (Hobbs *et al.*, 1993). In nopaline strains of *A.tumefaciens*, there is a single T-DNA molecule of about 24 kb which is inserted into the plant genome. In some octopine-type crown galls, two non-contiguous segments are found, the T_L and the T_R. T_L is present in all transformed cell lines and is functionally equivalent to the T-DNA found in nopaline cell lines. T_R (7 kb) is not found in all tumour lines and its copy number can differ from that of T_L, indicating an independent transfer process (Yadav *et al.*, 1982; Holsters *et al.*, 1982).

Crown gall tissue differs from normal plant tissue and T-DNA encoded genes are responsible for this difference (Binns and Thomashow, 1988). The growth of normal plant cells *in vitro* requires exogenously added phytohormones, but gall cells are able to synthesize their own (Binns and Thomashow, 1988). The gall cells acquire additional growth hormone genes from the T-DNA of the infecting *Agrobacterium* with encourages cells to divide. Another feature of crown gall cells is that they produce and excrete unique amino acid and sugar derivatives, called opines. The type of opine formed by crown gall cells depends on the Ti plasmid of the infecting

Agrobacterium strain. These substances are not formed by normal plant cells (Messens *et al.*, 1985). These are special nutritive substances required by *Agrobacterium* as a carbon, nitrogen and energy source (Drummond, 1979). Opines also promote the conjugal transfer of the Ti plasmid within a population of *A. tumefaciens* in the rhizosphere (Binns and Thomashow, 1988).

The infection of plants by *Agrobacterium tumefaciens* has been called "genetic colonization" and is a process of natural genetic engineering (Hooykaas and Schilperoort, 1992). The process of crown gall induction consists of a number of discrete, essential steps (Hooykaas and Schilperoort, 1992). Wounding of the plant is necessary to allow entrance of the bacteria and to make available plant wound compounds which induce the virulence system (Binns and Thomashow, 1988). The bacteria multiply in the wound sap and attach to the walls of the plant cells within the wound. Subsequently, the T-DNA is transferred and expressed in the plant cells even before full integration into the host genome. After integration, T-DNA expression is maintained and tumours develop at the wound site. Cell divisions are triggered by the continuous production of auxin and cytokinin by infected cells from T-DNA encoded enzymes. The T-DNA containing cells also produce and excrete opines that are consumed specifically by the infecting agrobacteria. Inciting bacteria need only infect a single cell of the plant, but by the induced multiplication of this cell, are able to obtain an abundant supply of opine substrate. Genes for the production of plant hormones, carried into the plant cell on the T-DNA, ensure that one transformed plant cell multiplies into a huge opine production centre, the gall (Messens *et al.*, 1985; Hooykaas and Schilperoort, 1992). The gall also gives physical protection to the inciting bacteria, as they may become enclosed within the gall tissue. Only the bacteria benefit out of this parasitic association and the plant gains nothing (Drummond, 1979).

Soil, the environment in which *Agrobacterium* resides, is a complex environment, and the first step in the crown gall infection process is the recognition by *Agrobacterium* of susceptible host plant tissue within the soil (Melchers *et al.*, 1989). Healthy plant roots exude a mixture of organic molecules into the soil which attract bacteria into the rhizosphere. However, when plants are wounded, they exude

pathway is activated when plants are injured, and this leads to the production of secondary metabolites, including phytoalexins, flavanoids and lignin precursors (Zambryski, 1988). These phenolic substances leaked into the soil act as chemoattractants for virulent *Agrobacterium* (Shaw, 1990). Polyphenolic substances act on *Agrobacterium* at several levels. Two of these levels require both the *virA* and *virG* loci, suggesting a multifunctional role for these genes. At low concentrations of polyphenolics, the chemotaxis of bacteria towards a wound site requires constitutive levels of *virA* and *virG* gene expression only. At higher levels of plant wound substances experienced by the bacterium as it moves towards the wounded tissue, the entire set of *vir* loci are fully activated allowing the gene transfer mechanism of *Agrobacterium* to proceed (Shaw, 1990). At very high concentrations of polyphenolics, bacterial motility is suppressed, preventing the bacteria leaving the wound site (Shaw, 1990). The host plant is also made competent for infection by *Agrobacterium* when it is wounded, as wounding encourages cell division and DNA replication and repair, all of which favour the integration of the T-DNA into the plant genome (Binns and Thomashow, 1988).

The next step in the infection process involves the binding of the *Agrobacterium* cells to the plant cell surface following chemoattraction (Binns and Thomashow, 1988). Studies of the infection process which involved washing the wounded plant tissue at different times following inoculation, showed that bacterial binding is a prerequisite for T-DNA transfer (Sykes and Matthysse, 1986). In the initial phase of attachment, a loose reversible binding of bacteria occurs after one hour. This is followed by the synthesis of cellulose fibrils by the bacteria until they become irreversibly bound to the plant surface. This has occurred approximately two hours after infection. Only after four hours, when the bacteria are firmly attached to the plant cells, does the T-DNA transfer process begin (Sykes and Matthysse, 1986).

The initial binding of bacteria to the plant cell surface is site specific. The bacterial receptor is thought to be ß-1-2 glucan, while the plant cell receptor is not known (Binns and Thomashow, 1988). Another bacterial protein called rhicadhesin is also thought to be involved in bacterial adhesion (Hooykaas and Schilperoort, 1992). Three bacterial chromosomal loci have been studied for their role in the attachment

(Cangelosi *et al.*, 1989). These three loci are constitutively expressed in *Agrobacterium* (Hooykaas and Schilperoort, 1992). *ChvA* and *chvB* are located on a 15.5 kb segment of the chromosome, while the *pscA* locus is approximately 3.0kb in size. The 8.5 kb *chvB* portion encodes a membrane protein that acts as an intermediate in the synthesis of cyclic ß-1,2 glucan. The *chvA* protein has high homology to bacterial and eucaryotic export proteins. The gene product of *pscA* has not been characterized, but mutations in this region result in bacteria which do not produce cellulose fibrils, do not make ß-1,2 glucan and are also non-motile (Binns and Thomashow, 1988). The *chvA*, *chvB* and *pscA* genes of *Agrobacterium* are also analagous to the *ndvA*, *ndvB* and *ecoC* loci of *Rhizobium meliloti* (Zambryski, 1988), showing that gene function is conserved amongst these closely related bacterial species.

Cells of *Agrobacterium* are also capable of producing a pectinase, thought to aid the bacterium in penetrating the plant cell wall (Matthysse *et al.*, 1981). The plant cell is not actively involved in bacterial attachment, as *Agrobacterium* binds equally well to dead plant cells (Matthysse, 1987).

The next stage of *Agrobacterium* infection involves the role of the virulence (*vir*) region in the transfer of T-DNA into the plant cell (Binns and Thomashow, 1988; Hooykaas and Schilperoort, 1992 and Mozo and Hooykaas, 1992). Products of the *vir* genes can act in *trans* to effect transfer of the T-DNA into the plant cell. This property of the *vir* region has been useful in developing vectors for plant cell transformation (Hooykaas and Schilperoort, 1992). The nucleotide sequence of each of the *vir* genes is known, and transcripts from each of the loci have been identified (Hooykaas and Schiperoort, 1992; Mozo and Hooykaas, 1992). Both the *virA* and *virG* genes are expressed constitutively at low levels in bacterial cells, prior to induction by plant wound substances. Following activation of the *virA* gene by phenolic substances, the *virA* gene product, located on the inner bacterial membrane, transmits a chemical signal that changes the *virG* gene to an induced form. The product of the induced *virG* gene then transcriptionally activates the expression of the remaining *vir* genes. Following the induction of the whole *vir* gene complex, the T-DNA is cut, transcribed, processed and transferred out of the bacterial cell. Single stranded (ss) molecules (coding and T-strand)

bottom strand of the T-region can be detected in induced *Agrobacterium* (Mozo and Hooykaas, 1992). Evidence has acccumulated in favour of a linear, single-stranded polar intermediate or "T-strand", the production of which is enhanced by the binding of the VirC1 protein to the right-border-proximal overdrive sequence. However, with lower frequency, double stranded (ds) T-molecules have also been detected. It is now known that the T-DNA is not transferred to plants in a circular form (Timmerman *et al.*, 1988; Toro *et al.*, 1989; Mozo and Hooykaas, 1992).

The formation of both ss- and ds-T molecules is dependent on the activity of two proteins called VirD1 and VirD2 which are encoded by the *virD* operon. These proteins together determine an endonuclease activity capable of nicking the border repeats at a precise site. The nick sites act as starting points for DNA synthesis in the 5' - 3' direction. The T-strands are then released by displacement. The nick sites within the border repeats define the DNA segment that is transferred to plant cells (Mozo and Hooykaas, 1992; Hooykaas and Schilperoort, 1992).

Agrobacterium does not introduce naked DNA into the plant cell. Recent evidence indicated that T-strands retain the VirD2 protein covalently attached to the 5' terminus, and that the presence of this protein makes the 5' end less vulnerable to exonuclease attack during transfer. The VirD2 protein may also act as a pilot to direct the T strand to the nucleus of the transformed plant cell, since it contains nuclear targeting sequences (Mozo and Hooykaas, 1992; Hooykaas and Schilperoort, 1992). The 69 kDa VirE2 protein encoded by the *virE* operon is a ssDNA-binding protein, which is able to coat the T-strands by cooperative binding, leading to long thin nucleo-protein filaments. However, the presence of such nucleoprotein complexes in transformed plant cells has not yet been demonstrated. The initial steps of T-DNA transfer is very similar to bacterial conjugation (Lichtenstein, 1987; Howard and Citovsky, 1990).

The final event in the transformation of plant cells by *Agrobacterium* is the integration of the T-DNA within the plant genome. Little is known of the DNA intermediates which exist within the plant nucleus before integration, although there are indications that circular molecules may be formed (Bakkeren *et al.*, 1987). The mechanism of T-DNA integration into the plant genome

(Matsumoto *et al.*, 1990). T-DNA insertion into the plant genome is random with respect to sequence specificity, but shows a definite affinity for open, transcribed chromatin regions (Herman *et al.*, 1990). There is currently no direct evidence for the involvement of any Ti-plasmid encoded proteins in the T-DNA integration step (Janssen and Gardner, 1989). T-DNA integration into the host genome is thought to be part of a plant integration mechanism normally used for mitosis related recombination events. Somehow, *Agrobacterium* has been able to make use of these existing host functions for its own survival purposes (Janssen and Gardner, 1989).

Following on integration of the T-DNA molecule into the plant genome, genes carried on the T-DNA are expressed, resulting in the transformed tumour phenotype and opine synthesis. The *onc* (oncogenicity) genes include *ipt*, *iaaM* and *iaaH* (Hooykaas and Schilperoort, 1992). Originally, two of these genes were called *aux* genes and a third, the *cyt* gene. The *aux* mutants induced shooty tumours on tobacco and kalanchoe, whereas the *cyt* mutants formed rooty tumours on these plants. Because of these characteristics, the *aux* genes were later renamed the *tms* (tumour morphology shoot) or *shi* (shoot inhibition) genes, and the *cyt* gene was renamed the *tmr* gene. These tumour phenotypes on tobacco corresponded to the response of the tissue to an excess of auxin or cytokinin, respectively, in *in vitro* tissue culture (Hooykaas and Schilperoort, 1992). Expression of the cloned *cyt* gene in *E.coli* showed that the protein encoded by this gene was an isopentenyl-transferase capable of catalysing the formation of a specific cytokinin, isopentenyl-AMP. Isopentenyl-AMP was formed from isopentenyl-pyrophosphate and AMP. Because of this reaction, the *cyt* gene is now often called the *ipt* gene (Hooykaas and Schilperoort, 1992).

When the two *aux* genes were cloned and transferred into *E.coli*, it was revealed that both these genes together mediated a pathway for the synthesis of the auxin indole-acetic acid (IAA). The *aux* genes, *aux-1* and *aux-2,* are now also known as *iaaM* and *iaaH* respectively. The overproduction of auxin and cytokinin by infected plant cells explains why crown gall cells proliferate even in the absence of externally applied phytohormones (Hooykaas and Schilperoort, 1992).

The molecular mechanism underlying the process of crown gall induction by *Agrobacterium tumefaciens* has been summarized in this section. When it was known that gene transfer from *Agrobacterium* to plants formed the molecular basis of crown gall induction, the next step was to manipulate this gene transfer system, for the genetic engineering of plants (Hooykaas and Schilperoort, 1992). A summary of the modification of the Ti plasmid and T-DNA is presented in the following section.

1.6 The development of plant genetic engineering systems based on the Ti plasmid of *Agrobacterium tumefaciens*.

Knowledge of the molecular processes whereby *Agrobacterium* transfers a defined segment of its own DNA into the plant genome has resulted in the development of Ti plasmid based gene transfer systems to transfer new genes into plants (Hooykaas and Schilperoort, 1992). Already the use of *Agrobacterium*-mediated gene transfer has resulted in fertile transformed plants being obtained for an ever increasing list of dicotyledonous plant species (Hooykaas and Schilperoort, 1992).

There are several general characteristics required by a gene vector system in order to successfully transfer new genes into plants. The system should be easy and efficient to use, and introduced foreign genes should be stably maintained in the plant genome and expressed. The gene vectors should also have a wide plant host range. In general, *Agrobacterium*-mediated gene transfer satisfies these requirements, but has a host range restriction. With few exceptions, *Agrobacterium*-mediated gene transfer is limited to dicotyledonous plants (Binns and Thomashow, 1988).

There are several key elements which are required of a gene vector plasmid to transfer genes from *Agrobacterium* to the plant nucleus. These are the T-DNA border repeat sequences and *vir* genes (Hooykaas and Schilperoot, 1992). It has been possible to construct gene vectors using the fact that any DNA artificially placed within the 25 bp border repeat sequences will be transferred to the plant cell and integrated into the plant genome (Armitage *et al.*, 1988). The

to the T-DNA for transfer to occur and can function in *trans* (Armitage *et al.*, 1988; Hooykaas and Schilperoort, 1992).

The wild-type T-DNA *onc* genes are not required for the transfer of the T-DNA to the plant cell, nor for the integration of T-DNA into the plant genome. They can be deleted from the T-DNA to create a T-DNA based vector with room for other desireable genes to be inserted. Also, as the *onc* genes produce plant hormones which prevent the transformed cells differentiating into a whole plant, the deletion of these genes allows the regeneration of plants from transformed tissues and cells (Armitage *et al.*, 1988; Hooykaas and Schilperoort, 1992).

The first plant gene vectors developed were Ti plasmids with the *onc* genes deleted from the T-DNA and replaced with sequences from the plasmid pBR323. Any pBR323 derived plasmid containing foreign genes could be integrated into the vector through homologous recombination (Zambryski *et al.*, 1982). These early plasmids, called cointegrate or *cis* acting plasmids, were large and cumbersome due to the presence of opine genes, pBR323 sequences and *vir* genes. These vectors were eventually replaced by newer vectors which contained only a minimum of T-DNA derived sequences. Examples of cointegrate vectors include pMON200, pGV1103 and pGV2260. The cointegrate vector pGV2260 can also function in a binary vector system (Armitage *et al.*, 1988).

The next generation of vector plasmids were called binary vectors. These vectors, which are now in common usage, were based on observations that the *vir* region does not need to be physically linked to the T-DNA to effect T-DNA transfer. In fact, the *vir* genes could be located on another replicon in the same cell and could still function (in *trans*) to effect T-DNA transfer (Herrera-Estrella and Simpson, 1988; Hooykaas and Schilperoort, 1992). The development of binary vectors was also based on certain plasmids (pUC and pBin19) which could replicate in both *E.coli* and *Agrobacterium*. This characteristic facilitates the manipulation of binary vectors. The plasmids can be isolated from *E. coli* in quantity using DNA maxipreparation methods. Using recombinant DNA technology, the plasmids can be altered and transferred back into *E.coli* using DNA transformation. From *E.coli*, the binary vectors can easily be transferred to *Agrobacterium*

conjugation.

The *Agrobacterium* strains used in *Agrobacterium*-mediated gene transfer are equiped with either a virulent or disarmed (helper) Ti plasmid. These Ti plasmids act as suppliers of the *vir* functions in *trans.* The binary vector plasmid itself supplies the T-DNA border sequences, the selectable marker genes and/or the desirable foreign gene (Ward and Barnes, 1989). Binary vector plasmids can also be designed so that the border sequences flank multiple cloning sites which allow insertion of foreign DNA. Examples of binary vectors are pBIN19 (Bevan, 1984), pRAL3940 (Hoekema *et al.*, 1985) and pL22 (Simoens *et al.*, 1986), as well as the pJIT vectors (Guerineau *et al.*, 1990) used in this study.

Both cointegrate and binary vector systems have certain advantages and disadvantages. Cointegrative vectors have the disadvantage that their efficiency of transfer relies on the success of bacterial conjugation as well as a cointegration event to construct the fully functional vector. The frequency of obtaining transconjugants is therefore low (Herrera-Estrella and Simpson, 1988). Mapping of the cointegrate is also required in order to confirm integration of the two vectors around the region of homology.

Binary vectors are small and easy to manipulate *in vitro* and because of this, have now largely replaced cointegrate vectors. The frequency of binary vector transfer to an *Agrobacterium* helper strain using triparental mating is high, and the procedure is uncomplicated (Herrera-Estrella and Simpson, 1988). A major disadvantage of binary vectors is that the conjugal transfer of these vectors from *E. coli* to *Agrobacterium* often results in plasmid rearrangements. If bacterial transformation of the vector plasmid into *Agrobacterium* is used instead of conjugal transfer, DNA rearrangements do not occur (An *et al.*, 1988). The two types of Ti plasmid based gene vector systems are depicted in Fig. 1.2.

The *Agrobacterium* transformation system based on the Ti plasmids of *A. tumefaciens* has a number of important limitations. Consequently, other systems have been designed to overcome these drawbacks. The greatest problem with the

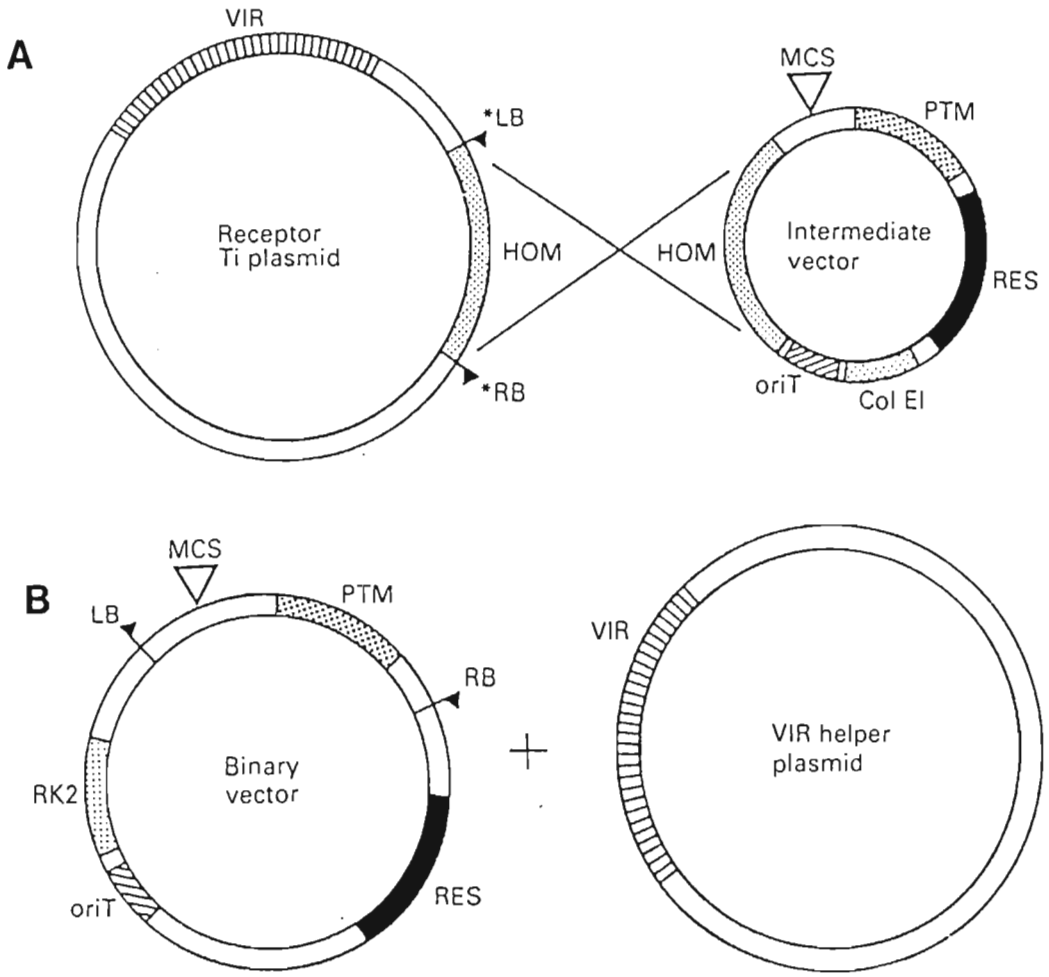

Fig. 1.2. Schematic diagram of generalized cointegrate (A) and binary (B) vector systems. VIR = virulence region; HOM = homologous regions within which recombination may occur for cointegration. LB, RB = left and right borders; MCS = multicloning site; PTM = plant transformation marker; RES = antibiotic-resistance marker to select for presence of vector sequences in bacterial host; oriT = origin of transfer and *bom* site for conjugative mobilization of vectors; Col E1 = origin of replication from plasmid Col E1; RK2 = wide host range origin of

monocotyledons, a group of plants which contains most of the world's staple food plants. Direct DNA transfer methods have been developed to fill this niche (Lindsey, 1992). These are usually physical methods which require only the means to introduce DNA directly into plant cells, protoplasts or into intact tissue. Simple, small gene vectors, or in some cases, sheared genomic DNA, can be transferred directly into plant cells using a variety of methods (Golz *et al.*, 1990). Such direct DNA methods include electroporation (use of electrical discharges) and the use of chemicals (polyethylene glycol) to introduce DNA into cells. Also, bombardment with DNA coated particles accelerated from a biolistic gun can introduce DNA into plant cells. The main disadvantage of most direct DNA transfer techniques is that plant protoplasts are required. Regeneration of fertile plants from protoplasts is not always a simple procedure. The particle gun method, however, has the advantage that it can transfer DNA coated particles into whole explants, including meristematic regions, which regenerate easily (Lindsey, 1992).

Also, the complexity of the *Agrobacterium*-mediated gene transfer system can make successful transformation difficult. In certain recalcitrant crop plant species, extensive optimization is often required to link transformation and adventitious shoot regeneration in tissue culture to produce transgenic plants. There is, therefore, a continuing search for less complex methods of transferring genes to plants (Armitage *et al.*, 1988).

The original attempts to express a number of bacterial or animal genes in a plant system using a Ti plasmid based system failed because plant cells could not recognize the foreign transcription signals (promoter sequences) carried by the foreign genes (Herrera-Estrella *et al.*, 1983). To overcome this problem, chimaeric genes were constructed with a constitutive *nos* (nopaline synthase) promoter from an *Agrobacterium* nopaline Ti plasmid. This resulted in foreign gene expression in plants cells and the translation of functional foreign proteins. Early studies placed the *nos* promoter behind the coding sequence of the neomycin phosphotransferase gene (*nptII*) and this is still in common use today in Ti plasmid based vectors (Herrera-Estrella and Simpson, 1983). However, the *nos* promoter is not a strong promoter in plants, and other more powerful promoters were later sought to

plant systems is a very active area of research at the present (Kuhlemeier, 1992).

One of the best studied plant promoters is the cauliflower mosaic virus (CaMV) 35S promoter (Benfey *et al.*, 1989; Benfey *et al.*, 1990; Terada and Shimamoto, 1990; Stefanov *et al.*, 1994). The 35S promoter was found to have a 30-fold greater activity than the *nos* promoter, and thus has found wide useage in plant genetic engineering (Odell *et al.*, 1987). The CaMV 35S promoter controlled the expression of the *sul* I and *uidA* genes in this present study.

The cauliflower mosaic virus is a double-stranded plant virus which contains two promoters responsible for producing transcrips of 35S and 19S RNA in infected plants. The molecular biology of this virus is well known (Covey *et al.*, 1990). The 35S RNA is the major RNA species produced by the virus and includes the complete viral genome (Pierce *et al.*, 1987; Covey *et al.*, 1990). Expression of the 35S promoter largely constitutive in transgenic plants and is active in most plant organs and at most stages of development (Benfrey *et al.*, 1987). The activity of the 35S promoter is also insensitive to various endogenous and environmental cues such as hormones, heat shock or light (Kuhlemeier, 1992). The 35S promoter can confer foreign gene expression in both dicotyledonous and monocotyledonous plants (Terada and Shimamoto, 1990), as well as being active in procaryotic cells. All these factors have made this promoter popular in plant genetic engineering.

The original Ti plasmid-based gene vectors did not contain selectable marker genes. Instead, transformed plant tissue was identified using a screening process. The first screenable markers were those derived from *Agrobacterium* T-DNA i.e. the opine synthase genes. Assays were developed to detect both nopaline and octopine in transformed plant tissues (Herrera-Estrella *et al.*, 1983; Reynaerts *et al.*, 1988). Most genetic engineering systems now rely on a specific dominant selectable marker to enable the recovery of transgenic plants.

The first selectable marker was the *nptII* gene which Herrera-Estrella *et al.* (1983) placed after a *nos* promoter and achieved the first foreign gene expression in plants. This gene has been generally incorporated into binary vectors (An *et al.*,

1985) and also has the advantage that it is a dominant gene in plants (Perl *et al.*, 1993). The *nptII* gene produces a neomycin phosphotransferase enzyme (NPTII) which inactivates a related group of aminoglycoside antibiotics, neomycin, kanamycin and geneticin (G418) by phosphorylation (Pazkowski *et al.*, 1984). Nevertheless, the use of the *nptII* gene has several drawbacks. Many important crops plants possess low sensitivity to kanamycin and transformants are not efficiently selected. Also, in some cases, the extent of kanamycin resistance is not well correlated with the expression level of the gene of interest, co-transformed with the *nptII* gene (Perl *et al.*, 1993). There is therefore a continual search for new genetic markers that can be used in plant genetic engineering to select for transgenic cells, tissues or plants.

Resistance genes for other antibiotics are also used as selectable marker genes. The hygromycin phosphotransferase (*hpt*) gene can be transformed into plants, but selection in leaf disc experiments is not as effective as selection using kanamycin (Gatz *et al.*, 1991; Gotz *et al.*, 1990; Reynaerts *et al.*, 1989). Other selectable marker genes encoding resistance to chlormaphenicol, methotrexate, streptomycin, bleomycin, sulfonamide antibiotics, bromoxynil, 2,4-dichlorophenoxyacetic acid, glyphosate and phosphinothricin (Perl *et al.*, 1993) are in use. A new marker system involves selecting transformed plants on the basis of a chimeric gene encoding a bacterial desensitized aspartate kinase gene. Transformants are selected on the basis of their resistance to the presence of lysine plus threonine in the regeneration and rooting media. Similarly, plants transformed with a chimeric gene encoding a bacterial dihydrodipicolinate synthase can be selected for on the basis of resistance to the toxic lysine analogue S-aminoethyl L-cystein (Perl *et al.*, 1993).

As well as selectable marker genes, six screenable reporter genes can be used to indicate the presence and expression of a foreign gene derived from genetic engineering (Herrera-Estrella *et al.*, 1989). Genes coding for opine synthesis, including *ocs* (octopine synthase gene) and *nos* (nopaline synthase gene), chloramphenical acetyltransferase (CAT) (Töpfer *et al.*, 1988), ß-glucuronidase (GUS) (Jefferson *et al.*, 1987) and firefly luciferase (Howell *et al.*, 1989) have been used extensively to analyze the activity and regulation of plant genes and

promoters, and to follow the successful transformation of plant tissue in genetic engineering. However, it is the GUS system which has found the most widespread use as a screenable reporter system in plant genetic engineering (Jefferson *et al.*, 1986; Jefferson *et al.*, 1987; Jefferson, 1987).

The GUS reporter gene system was developed from a cloned *E. coli* ß-glucuronidase (*uidA*) gene. The enzyme ß-glucuronidase (GUS) is a stable enzyme and will tolerate a wide variety of conditions, and may be assayed at any physiological pH, with a pH optimum between 5.2 and 8.0. ß-glucuronidase is also reasonably resistant to thermal inactivation, with a half-life of about 2 hours at 55 ° C. It is generally thought that there is very little or no detectable endogenous ß-glucuronidase activity present in a wide variety of plant, yeast and animal tissues (Jefferson *et al.*, 1987; Jefferson, 1987). However, Hu *et al.* (1990) and Kosugi *et al.* (1990) report that an intrinsic GUS-like activity exists in many plants, including *Nicotiana tabacum*. The use of methanol in the GUS reaction mixture eliminates this endogenous activity in plant tissues (Kosugi *et al.*, 1990).

ß-glucuronidase is a hydrolase which catalyzes the cleavage of a wide variety of water-soluble substrates. Many of these are available commercially as spectrophotometric, fluorimetric and histochemical substrates. Commercially available substrates for ß-glucuronidase include 4-methyl umbelliferyl glucuronide (MUG) for the fluorimetric assay, X-gluc (5-bromo-4-chloro-3-indolyl ß-D-glucuronide) for histochemical analysis and p-nitrophenyl glucuronide (PNPG) for spectrophotometric analysis. Either rapid qualitative assays or sensitive quantitative assays can be performed (Jefferson, 1987).

1.7 Origins, cultivation and breeding of commercial tobacco (*Nicotiana tabacum* L.).

Tobacco was one of the many commodities from pre-Columbian South America brought back to the Old World by early European explorers. Following the introduction of tobacco plants to Europe in the 1550's, the plants were grown for medicinal and ornamental purposes, but quickly became of economic importance as a source of smoking and chewing tobacco (Koshhar, 1991).

Commercial tobacco is manufactured from two *Nicotiana* species belonging to the plant family Solanaceae, *Nicotiana tabacum* L. and *Nicotiana rustica* L. (McIlroy, 1963), Cultivated *Nicotiana tabacum* probably originated from natural hybridization between *Nicotiana sylvestris* Speg and Comes (2n = 24) and *Nicotiana otophora* Grisebach (2n = 24) (Kochhar, 1981). The resulting sterile hybrid became fertile after natural chromosome duplication, to give rise to the present day *Nicotiana tabacum* (2n = 48). There is some speculation that *Nicotiana tomentosiformis* Goodspeed, and not *Nicotiana otophora*, could be one of the progenitors (Kochhar, 1981). The original hybridization must have been a rare event because *Nicotiana tabacum* has never been found in the wild, except as an escape from cultivation. *Nicotiana rustica*, like *Nicotiana tabacum*, is also an amphidiploid (2n = 48), resulting from a natural hybridization event between two wild species, *Nicotiana undulata* Ruiz and Pavon, and *Nicotiana paniculata* L., with a subsequent doubling of the chromosome number (Kochhar, 1981).

The breeding of commercial tobacco for improvement is difficult because the plant readily self-pollinates and therefore it is difficult to obtain hybrids. The genetic base for improvement is also not large. In tobacco, the quality of the leaves, the final commercial product, is very important. For this reason, the introduction of new germ plasm into existing cultivars through cross-hybridization is not lightly undertaken in case commercially important leaf quality is lost. Genetic engineering could help to introduce new traits into valuable cultivars without any change to the existing traits (P. Nelson, pers. comm.).

Nicotiana tabacum occupies nearly 90% of the world's tobacco acreage, and is largely used for making cigarettes and cigars because of its low (4 - 6%) nicotine content. Despite health warnings, 1.5 billion people around the world use tobacco regularly. *Nicotiana rustica* is of secondary importance, and tobacco made from this species is used only for hookah smoking, for chewing and for snuff. China, the United States of America (USA), India, Brazil, Turkey, Bulgaria, Japan, Indonesia, Italy, Greece, Canada, Thailand and Zimbabwe are the major tobacco producing countries of the world. The USA, Zimbabwe, Malawi, Turkey, Greece and India are the leading exporters of tobacco to the world ("The Tobacco Industry",

The annual value of the South African tobacco crop, measured in gross agricultural production, is in the region of R300 million. Approximately 25000 hectares are under cultivation in South Africa annually. The tobacco industry stimulates the South African economy through its effect as an employer of over sixty thousand people, as well as through its support of the chemical and packaging industries. The tobacco industry also yields a significant return on investment for primary producers and co-operatives. Significant revenue is generated annually for the South African government through excise duties and value added tax (South African Tobacco Board annual report, 1993).

Despite the size of the tobacco industry in South Africa, the country is not self-sufficient in tobacco, and imports are needed annually to supplement local production. Despite the negative publicity that tobacco smoking is currently receiving in First World countries, the local demand for tobacco is increasing at a rate of 3% per annum. Due to the recent drought, the style of tobacco produced has changed to a low nicotine, high tar type. Increased imports of low tar tobacco have been necessary for blending with locally grown tobacco, to supply the market demand for low tar tobacco (A.I.F. Cornelissen, pers. comm.).

1.7.1 Cultivation of commercial tobacco in South Africa.

Tobacco has been grown in South Africa since 1882 and today has come to be an important crop and industry (South African Tobacco Board annual report, 1992). The tobacco growing areas of South Africa are located in the following regions: Northern Transvaal, North West, Eastern Transvaal, the Orange Free State, Eastern Cape, Western Cape, and in parts of KwaZulu/Natal (Fig. 1.3). In South Africa, seedlings are cultivated in seed beds between May and September, and transplanted out to fields from October to January. The growth cycle of tobacco, from sowing to harvesting the leaves, requires 18 months ("The Golden Leaf", Tobacco Control Board publication, 1970). In South Africa, most tobacco is grown under irrigation at high input cost.

The Tobacco and Cotton Research Institute, located near Rustenburg, about 100 km west of South Africa's capital city, Pretoria, carries out

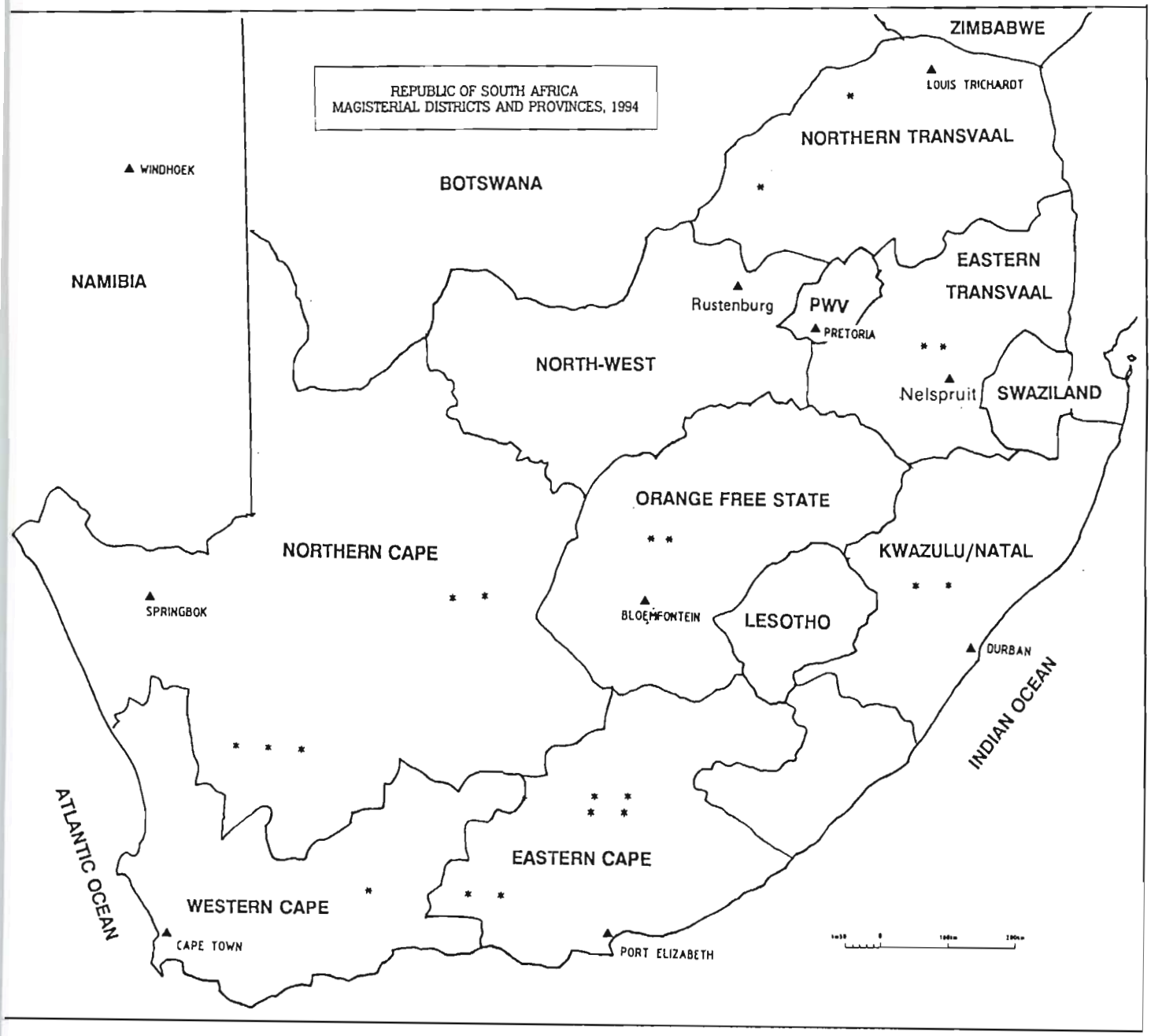

Fig. 1.3. Map of the Republic of South Africa. The areas where tobacco is cultivated commercially are indicated by asterisks.

activities for the breeding, agronomy, production and curing of tobacco, and has undertaken to make the most suitable tobacco cultivars for local growing conditions available to farmers (Tobacco Research Institute booklet, undated). The Institute is the official producer of tobacco seed, and about 2000 kg are sold annually to tobacco growers. The Institute concentrates on breeding tobacco for good leaf quality, for disease resistance, and for adaptation to local conditions (Tobacco Research Institute booklet, undated; M.C. Dippenaar, pers. comm.).

The cultivar TL33, released over twenty years ago, is the most important tobacco cultivar grown in South Africa at present. It is adapted to a wide range of localities and climatic conditions. It is also resistant to powdery mildew (*Erysiphe cichoracearum*), tobacco mosaic virus (TMV) and to race one and two of black shank disease (*Phytophthora parasitica* var. *nicotianae*). The cultivar 20/19 is also extensively grown in South Africa, especially in the Nelspruit area about 400 km east of the capital city, Pretoria. This cultivar is resistant to Granville Wilt (*Pseudomonas tabacum*). Another important cultivar is A4, grown in the Loskop area east of Pretoria. This cultivar is highly resistant to black shank disease. However, it is very susceptible to Granville Wilt disease, which limits its use. Also, the leaf is not of high quality. The cultivar J6 is very similar to A4 and is grown in the Transvaal lowveld around Nelspruit. All these tobacco cultivars are classed as Virginia tobacco. Oriental tobacco is grown in small amounts in the Western Cape. Burley tobacco is no longer grown in South Africa (M.C. Dippenaar, pers. comm.; A.I.F Cornelissen, pers. comm.).

1.7.2 The relevance of plant genetic engineering to the South African tobacco industry.

The use of genetic engineering technology is currently being applied to tobacco improvement in many countries around the world. Many of the large international multinational agrochemical companies, as well as smaller biotechnology companies, are developing transgenic tobacco lines for commercial release (Beer, 1989). Du Pont, Agrecetus and Plant Genetic Systems (PGS) have developed herbicide resistant tobacco, while PGS and Rohm and Haas have developed insect resistant tobacco cultivars using genetic engineering techniques. Lubrizon, Monsanto and Agricultural Genetics Company have also developed virus resistant transgenic tobacco cultivars (Beer, 1989).

The application of a long term strategy involving genetic engineering could assist the South African tobacco industry to increase the production of tobacco in South Africa. New genes introduced through genetic engineering could increase disease resistance, allowing tobacco to be cultivated more extensively with less yield loss due to disease. Tobacco cultivars transformed for herbicide resistance could also result in increased production through the efficient removal of competing weeds, especially at the seedling stage.

In the longer term, it is expected that health regulations will force a decrease in tobacco smoking internationally, but this need not bring about a decrease in commercial tobacco cultivation. As the technology already exists to transfer genes for heterologous proteins into tobacco, there are now indications that tobacco could be used as a manufacturing system to produce valuable pharmaceutical proteins, antibodies and peptides on an agricultural scale ("pharming") (Hiatt *et al.*, 1989; Hiatt, 1990; Tavladoraki *et al.*, 1993). Tobacco is able to accumulate relatively high concentrations of protein, exceeding 1% of total leaf protein (Swain, 1991). The practice of "pharming" with tobacco could be a viable business option for many of the hundreds of tobacco farmers in South Africa. This route would require a considerable input of funding both from local and international sources to obtain foreign genes for these products, carry out the genetic engineering and field testing, and to set up the processing infrastructure [illegible]

market the products. However, the end result may be that a significant and lucrative niche market exists for the more progressive tobacco growers in South Africa in the future.

1.8 Aims of this study.

This study developed out of an investigation into the methods by which transgenic herbicide resistant tobacco plants could be produced. The main aims, therefore, were to investigate the value of techniques currently available for inserting a herbicide resistance gene into tobacco, and to investigate the expression of this gene and accompanying marker genes in the transformants and the inheritance of these genes by progeny. As the focus of this study was on the commercial aspects of plant genetic engineering, South African commercial tobacco cultivars, rather than laboratory cultivars, were favoured. These cultivars had not previously been genetically engineered. Also, the tobacco industry is a large and important agricultural industry in South Africa and could benefit from the judicious use of plant genetic engineering.

Asulam is a herbicide which is not widely used in South Africa, but transgenic tobacco plants carrying the asulam resistance gene (*sul* I) clearly demonstrated in this study the effectiveness of foreign genes in protecting tobacco from herbicide action. The *sul* I gene is an unusual and seldom reported herbicide resistance gene. The effectiveness of the *sul* I gene as a selectable marker gene in plant genetic engineering is recognized (Yoder and Goldsbrough, 1994), and was evident from this study. The *sul* I gene also proved to be an excellent model gene to study foreign gene expression in transgenic plants. Once transgenic tobacco plants were obtained, they were utilized as a model system to learn more about foreign gene expression and inheritance in transgenic plants. The characteristics of the cauliflower mosaic virus (CaMV) 35S promoter used to drive the *sul* I and *uidA* genes was investigated in the transgenic tobacco plants. The implications of the tissue specificity, developmental regulation and stress regulation of the CaMV 35S promoter on the use of transgenic plants outside the laboratory is discussed.

Also considered in this study were the commercial aspects of plant genetic

engineering, the role of plant genetic engineering in present and future agriculture and the implications of biotechnology for Third World agriculture.

It must be emphasized that no field testing or commercialization of the transgenic South African tobacco plants produced during this study is planned. Any discussion on field testing is purely hypothetical and for the sake of this thesis.

Chapter 2

Preparation and analysis of *Agrobacterium tumefaciens* transconjugant strains for the genetic engineering of South African commercial tobacco cultivars.

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Preparation and analysis of *Agrobacterium tumefaciens* transconjugant strains for the genetic engineering of South African commercial tobacco cultivars.

2.0 Summary

The binary vector, pJIT119, was provided by the Plant Gene Toolkit Consortium, Norwich, United Kingdom, for experimental purposes. This vector carried a chimaeric *sul* I gene which conferred resistance to the sulfonamide herbicide asulam (Maybaker) when expressed in bacteria and plant cells. The vector also contained an *nptII* and *uidA* gene. pJIT119 was introduced into several *Agrobacterium tumefaciens* helper strains by triparental mating. The *Agrobacterium* transconjugant strain C58C1(pGV2260)(pJIT119) was analyzed in detail using Southern blot analysis before it was used to transform South African tobacco cultivars. The plasmid pJIT58/1 was obtained from Dr Karl Kunert of the John Innes Institute, Norwich. This plasmid was extracted in quantity from *E.coli*(pJIT58/1) and used in direct DNA transfer experiments. The vector pGV941 was obtained from Plant Genetic Systems (PGS) in Ghent, through the generosity of Dr Johan Peleman, and was used to provide a T-DNA probe for Southern blotting.

2.1 Introduction

Plant genetic engineering vector plasmids have been designed so that they are able to replicate in both *Escherichia coli* and *Agrobacterium*. This ability allows for plasmid DNA manipulation to be carried out in *E. coli*, after which the plasmid may be mobilized into a non-oncogenic *Agrobacterium* helper strain, which is used to transform plant cells. Transfer of the vector plasmid from *E. coli* to *Agrobacterium* can be achieved in several ways. The vector DNA can either be isolated in pure form from *E. coli* and transformed (Hinchee *et al.*, 1988) or electroporated (Mozo and Hooykaas, 1992) into *Agrobacterium*, or the vector plasmid can be mobilized from *E. coli* into *Agrobacterium* using bacterial conjugation (Armitage *et al.*, 1988; Rogers *et al.*, 1988).

Triparental mating is the most convenient method of carrying out a bacterial conjugation (Armitage *et al.*, 1988). Triparental mating requires a conjugation "helper" function, usually provided by the plasmid pRK2013 contained in an *E.coli* mobilizing strain. The other components required are a donor *E. coli* strain containing the vector plasmid and a recipient *Agrobacterium* strain (Ditta *et al.*, 1980; Armitage *et al.*, 1988). The choice of helper plasmid must be made specifically for the type of replicon present on the vector plasmid. The conjugation helper plasmid provides mobilization (*mob*) and transfer (*tra*) functions for the binary vector in *trans.* The binary vector transferred into a recipient *Agrobacterium* strain by bacterial conjugation must contain a specific origin of transfer (*oriT*) and an activation site (*bom*). The *tra* and *mob* gene products act on these sites to bring about transfer of the binary vector into the *Agrobacterium* strain (Armitage *et al.*, 1988).

For the genetic engineering of South African tobacco cultivars, two *Agrobacterium tumefaciens* strains, C58C1 Rif^r (Van Larebeke *et al.*, 1974) and LBA4404 (Hoekema *et al.*, 1983) were chosen as recipients for vector plasmids. These strains are known to transform tobacco (Draper *et al.*, 1988). The *Agrobacterium* strain C58C1 Rif^r is a disarmed strain of C58, cured for pTiC58 (Van Larebeke *et al.*, 1983). C58C1 Rif^r strains were used containing either the disarmed helper Ti plasmids pMP90 (Koncz and Schell, 1986) or pGV2260 (Deblaere *et al.*, 1985). The *Agrobacterium* strain,

LBA4404, is an octopine strain with an Ach5 chromosomal background, and harbours the disarmed Ti plasmid pAL4404. The plasmid pAL4404 functions as a helper Ti plasmid in a binary vector system (Hoekema *et al.*, 1983).

The helper Ti plasmid pMP90 forms part of a binary vector system. In the construction of pMP90, the T-DNA was deleted from pTiC58, but an intact *vir* region remains which functions in *trans* to effect T-DNA transfer (Koncz and Schell, 1986). The helper Ti plasmid pGV2260 was derived from an octopine Ti plasmid, pTiB6S3 (Deblaere *et al.*, 1986). The T-DNA region was deleted from pRiB6S3 and replaced with pBR322. The plasmid pGV2260 can be used as the helper Ti component of both binary and cointegrate vector systems. The pBR322 sequences function only in the cointegrate system and are not required when this plasmid is employed in a binary system. The plasmid pGV2260 also contains an intact *vir* region which supplies the functions necessary for T-DNA transfer. The *vir* genes act in *trans* in a binary vector system, and in *cis* in a cointegrate system (Armitage *et al.*, 1988).

For efficient transformation of plant cells using an *Agrobacterium*-mediated gene transfer, the optimum combination of helper Ti plasmid, *Agrobacterium* chromosomal background and vector is required (Armitage *et al.*, 1988; Van Wordragen *et al.*, 1991; Liu *et al.*, 1992). This relationship must be determined by experimentation for each new plant species or plant cultivar to be transformed (Byrne *et al.*, 1987; Draper *et al.*, 1988; Owens and Smigocki, 1988; Van Wordragen *et al.*, 1991). When the plant species or cultivar has been genetically engineered previously, the literature can be consulted for an appropriate combination of *Agrobacterium* strain and helper Ti plasmid.

The plant gene vector pJIT119 (18kb) is based on pBin19 and encodes resistance to the sulfonamide herbicide, asulam (Maybaker), through a gene (*sul* I) encoding a modified dihydropteroate synthase (Guerineau *et al.*, 1990). The modified enzyme is insensitive to inhibition by sulfonamides. Dihydropteroate synthase is an enzyme of the folic acid synthesis pathway in plants and bacteria. In pJIT119, the *sul* I coding sequence is fused to a pea ribulose bisphosphate carboxylase/oxygenase

(RUBISCO) transit peptide sequence. In plants transformed with pJIT119, the transit peptide sequence enables the *sul* I gene product to be transported to the chloroplast stroma, where it complements the endogenous, asulam-sensitive dihydropteroate synthase. The *sul* I gene in pJIT119 has been constructed with two cauliflower mosaic virus 35S promoters, giving greater expression in plant cells (Guerineau *et al*., 1990). The vector pJIT119 also contains a GUS (*UidA*) reporter gene under the control of a single CaMV 35S promoter, and a *nos-nptII* (neomycin phosphotransferase) selectable marker gene (Table 2.2).

The vector plasmid pJIT58/1 (5.2 kb) is based on plasmid pJIT58 containing a pUC replicon (Plant Gene Toolkit manual, 1988). The plasmid pJIT58/1 contains the GUS (*uidA*) reporter gene under the control of a single CaMV 35S promoter. However, it does not posses a *nptII* gene and cells or tissues transformed with this vector cannot be selected using kanamycin (Table 2.2).

The plasmid, pGV942 (14.2 kb), also functions in a binary vector system. It contains T-DNA border sequences for integration into the plant genome, and a *nos-nptII* gene to assist in the selection of transformed plant cells using kanamycin (Deblaere *et al*., 1987). In this study, pGV941 was used as a source of T-DNA for the construction of a radioactively labelled DNA probe. A *Hin*d III/*Eco* RI digest of this plasmid yields a 2.7 kb fragment containing the entire T-DNA including both borders (Table 2.2).

In this section the plasmids, pJIT119, pGV941 and pJIT58/1 were mapped with restriction enzymes to confirm that their structure corresponded to the vector maps supplied. Also, transconjugant *Agrobacterium* strains containing pJIT119 were prepared using triparental mating. The resulting transconjugant strains were analyzed prior to being used in an *Agrobacterium*-mediated leaf disc experiment to transform South African tobacco cultivars.

2.2 Materials and Methods

2.2.1 Bacterial strains and vector plasmids. The *Agrobacterium tumefaciens* and *Escherichia coli* strains used in this study and their characteristics are described in Table 2.1. Vector plasmids used in this study and their properties are listed in Table 2.2. The *Agrobacterium* and *E.coli* strains were maintained on Luria agar (LA) containing appropriate antibiotics. The *Agrobacterium* cultures were incubated at 28°C and the *E. coli* cultures were incubated at 37°C, both in floor standing incubator shakers.

2.2.2 *E. coli* plasmid DNA preparation. Plasmid DNA for the restriction endonuclease mapping of pJIT119 and pJIT58/1 was extracted from *E. coli* strains using the large scale method of Birnboim and Doly (1979). A single colony of each *E.coli* strain containing a vector plasmid was placed into 5 ml Luria broth (LB) containing selective antibiotics and shaken overnight at 37°C. The broth culture was subcultured by transferring 50 μl into 500 ml LB containing antibiotics and shaken overnight. The bacterial cells were collected by centrifugation at 8000 rpm for 5 minutes (4°C). The supernatant was poured off and the cell pellet resuspended in 20 ml of lysis solution (Birnboim and Doly, 1979) and incubated on ice for 5 min. Eighty milliliters of alkaline sodium dodecyl sulfate (SDS) (Birnboim and Doly, 1979) was added and the mixture incubated on ice for 4 min. To the cell lysis mixture, 60 ml of 3 M potassium acetate, pH 5.2 was added and mixed well. The lysis mixture was then centrifuged at 8000 rpm for 10 minutes and the supernatant poured through a plug of polypropylene wool into a clean 250 ml centrifuge tube. To precipitate the DNA, 100 ml propan-2-ol (isopropanol) was added. The solution was mixed well and centrifuged at 8000 rpm for 10 minutes. The resulting DNA pellet was rinsed first with 70% ethanol and finally with ether (Birnboim and Doly, 1979).

After evaporation of the ether in a fume hood, the DNA pellet was dissolved in 10 ml TE (Sambrook *et al.*, 1989). The volume was adjusted by weight. The resuspended pellet in 10 ml TE was weighed in the centrifuge tube (known weight) and the volume adjusted with TE buffer until the resuspended DNA pellet weighed 17.5 g. Then 20.79 g cesium chloride and 3.5 ml ethidium bromide solution

Table 2.1. Bacterial strains used in this study

STRAINS	DESCRIPTION OF STRAINS	REFERENCE
Escherichia coli		
HB101	Highly transformable strain used for large-scale plasmid production	Sambrook *et al.* (1989)
HB101(pRK2013)	HB101 containing pRK2013, a plasmid providing helper functions for bactrial conjugation	Ditta *et al.* (1980)
JM101	*E. coli* strain that will support the replication of binary vectors	Sambrook *et al.* (1989)
JM101(pJIT119)	JM101 containing the binary vector pJIT119	Plant Gene Toolkit Consortium, Norwich
JM101(pJIT58/1)	JM101 containing the binary vector pJIT58/1	Karl Kunert, John Innes Institute, Norwich
JM101(pGV941)	JM101 containing the binary vector pGU941	Deblaere *et al.* (1987)
Agrobacterium tumefaciens		
C581Rif^r	C58 Cured for pTIC58	Van Larebecke *et al.* (1974)
C58C1Rif^r(pMP90)	C58C1Rif^r containing the binary vector pMP90	Koncz and Schell (1986)
C58C1Rif^r(pGV2260)	C58C1Rif^r containing the binary vector pGV2260	Deblaere *et al.* (1986)
LBA4404(pAL4404)	Ach5 containing the helper Ti plasmid pAL4404	Hoekema *et al.* (1983)

Table 2.2. Plasmids used in this study.

PLASMID	DESCRIPTION	REFERENCE
pGV2260	A non-oncogenic helper Ti plasmid with T-DNA deleted and replaced with pBR322. Derived from pTiB6S3.	Deblaere *et al.*, 1986
pMP90	A non-oncogenic helper Ti plasmid derived from C58.	Koncz and Schell, 1986
pJIT119	Binary vector plasmid encoding *sul* I, *uidA* and *nptII*. Based on pBIN19.	Mullineaux, P. Plant Gene Tool Kit Consortium, Norwich, UK.
pJIT58	pUC based plasmid encoding *uidA*.	Mullineaux, P. Plant Gene Tool Kit Consortium, Norwich, UK.
pJIT58/1	Based on pJIT58, *kpn* I site replaced with a *Bgl*II site.	Kunert, K. Plant Gene Tool Kit Consortium, Norwich, UK.
pGV941	Based on the replicative function of pVS1, a 30Kb *Pseudomonas aeruginosa* plasmid. Encodes *nptII*.	Deblaere *et al.*, 1987
pRK2013	Conjugative plasmid used to mobilize binary vectors from *E.coli* to *A.tumefaciens* in a triparental mating. It has the *tra* genes of RK2. Carries a kanamycin resistance gene.	Ditta *et al.*, 1980
pGS9	Plasmid encoding *nptII*. Used for assay of bacterial NPTII.	Cabanes-Bastos *et al.*, 1987
pJIT85	pBIN19 based plasmid. Encodes *pat* gene (basta resistance).	Plant Gene Tool Kit Consortium, Norwich, UK.
pBI121	Based on pBIN19, encodes *uidA* gene.	Clontech Laboratories, Inc.

(5 mg/ml in distilled water) was added. The final CsCl density was 1.55 g/ml. Quickseal (Beckman) tubes were filled with the DNA preparation and sealed. The plasmid DNA was purified by centrifugation overnight at 55 000 rpm. After centrifugation, the plasmid DNA bands were visualized under UV light (350 nm). The collection of the plasmid DNA was achieved by puncturing the Quickseal tubes with a syringe needle attached to a small syringe and drawing off the plasmid DNA. Ethidium bromide was removed by three extractions with equal volumes of CsCl-saturated isopropanol (Sambrook *et al.*, 1989). The CsCl was removed from the DNA solution by overnight dialysis against sterile distilled water at 4 ° C. The plasmid DNA was precipitated by the addition of 0.1 volume of 2 M sodium acetate pH 5.6 and three volumes of ethanol. After careful mixing, the DNA solution was placed at -70 ° C for 30 minutes to precipitate the DNA. The precipitated DNA was pelleted by centrifugation at 10 000 rpm. The final DNA pellet was dried under vacuum and redissolved in 20 - 50 μl of 10 mM Tris-HCl pH 7.5. The DNA concentration was estimated following agarose gel electrophoresis by comparison with lambda DNA of known amounts.

2.2.3 *Agrobacterium tumefaciens* plasmid DNA preparation. Plasmid DNA extraction from selected *Agrobacterium tumefaciens* transconjugant strains containing the vector plasmid pJIT119 was carried out. The method of Casse *et al.* (1979) was used. To start a culture, a single colony of *A. tumefaciens* was added to 500 ml LB medium and the culture grown for two days at 28 ° C in a floor-standing orbital shaker. To pellet the cells, the culture was centrifuged at 5000 rpm for 10 min. The cell pellet was resuspended in TE and centrifuged (first wash). This second pellet was resuspended in TE containing 0.1% sodium sarkosyl before being centrifuged again at 5000 rpm (second wash). The pellet was resuspended in TE at 100 mg cells/0.5 ml TE. The centrifuge bottles were weighed before the addition of culture and were weighed again after the centrifugation of the culture, to determine the weight of the pellet. After resuspending the pellet in TE, the cell suspension was added to the lysing solution (Casse *et al.*, 1979) in an Ehlenmeyer flask, while swirling the flask gently for 60 seconds. To 190 ml of lysing solution, 10 ml of cell suspension was added. To completely lyse the bacterial cells, the mixture was allowed to stand at room temperature for 20 mins, and was occassionally swirled.

After 20 minutes, 6% v/v 2 M Tris, pH7.5 was added and the mixture swirled for 30 seconds. Enough 5 M NaCl was added to give a 3% solution, and the mixture allowed to stand for another 20 minutes. An equal volume of phenol saturated with 3% NaCl was added and the mixture swirled strongly for three minutes. The layers were separated by centrifugation at 5000 rpm for 20 minutes (Casse *et al.*, 1979). To remove the aqueous layer containing the DNA, the blunt end of a 10 ml glass pipette was applied to the surface and the aqueous layer drawn up into the pipette using an automatic pipette.

To precipitate the DNA from the aqueous solution, two volumes 96% ethanol were added and the mixture chilled at - 20°C overnight. The DNA was collected by centrifugation at 5000 rpm for 35 min. Following centrifugation, the ethanol was decanted and the DNA dried in a laminar flow cabinet. The DNA was resuspended in a small volume of TE. The concentration of this DNA was not determined. The crude DNA preparation was electrophoresed on 0.8% Seekem agarose gels for Southern analysis (Sambrook *et al.*, 1989).

2.2.4 Restriction enzyme mapping of vector plasmids. The mapping of pJIT119 and pJIT58/1 was accomplished using single enzyme digests. Restriction enzyme digests were carried out in the compatible buffers required for each enzyme. Digestion volumes were routinely 20 μl and contained 300 - 500 ng of DNA. Digestions were performed for one hour at 37°C using one unit of restriction enzyme. For electrophoretic analysis, 5 μl of DNA loading buffer (Sambrook *et al.*, 1989) was added to the 20 μl digestion volume and the sample loaded into individual wells on a Seakem 0.8% agarose gel. Agarose gel electrophoresis was carried out using an American Bionetics, Inc. horizontal submerged mini-gel system. Tris borate EDTA (TBE) buffer was routinely used in electrophoresis (Sambrook *et al.*, 1989). The DNA was electrophoresed at 96 V for 2.5 hours. DNA bands were detected using a UV transilluminator following ethidium bromide (5 mg/ml) staining of the agarose gels for two hours. The gels were photographed using a Polaroid CU-5 Land camera fitted with a fixed focal length attachment. Polaroid type 667 film (ASA 3000) was used with an exposure time of 8 sec at f4.7.

Restriction endonuclease enzymes used to map pJIT119 were *Sal* I, *Pst* I, *Eco* RI, *Xba* I and *Hind* III. To map pJIT58/1, *Xba* I and *Bam* HI were used. In both cases, the restriction enzymes chosen were key enzyme sites selected from each of the plasmid maps. The analysis of the enzyme digests was carried out using agarose gel electrophoresis. The DNA fragment size in each digest was determined by comparision with the lambda molecular weight marker III (Boehringer Mannheim).

To assist in determining the size of fragments resulting from each digestion, a standard curve was prepared for each agarose gel. Lambda molecular weight marker III (Boehringer Mannheim) was used for the standard curve. The distance (mm) that each DNA band travelled in 0.8% agarose following electrophoresis was plotted against the log of the size (bp) of each band. Using this graph, the size of DNA fragments resulting from the restriction enzyme digestions of pJIT119 and pJIT58/1 could be determined. These sizes were compared with information estimated from the plasmid maps and from a key provided in the manual of the Plant Gene Toolkit Consortium.

2.2.5 Preparation of *Agrobacterium tumefaciens* transconjugant strains. Triparental mating through bacterial conjugation was carried out to transfer vector plasmids from the *E.coli* donor strains (containing the vector plasmids) to the *Agrobacterium* recipient strains (Armitage *et al.*, 1988). The mobilizing strain was HB101(pRK2013). Luria broth was used to culture both the *Agrobacterium tumefaciens* and the *E.coli* strains. Single colonies of donor and mobilizing *E.coli* strains were inoculated into separate 5 ml amounts of LB broth with selective antibiotics and the cultures shaken overnight at 37°C in a floor standing incubator shaker. Single colonies of recipient *Agrobacterium tumefaciens* strains were inoculated into 5 ml of LB broth and shaken overnight at 28°C. Each of the three strains to be mated (ie. the donor *E. coli* strain, mobilizing *E. coli* strain and recipient *Agrobacterium* strain) were added to the surface of a single LA plate. One hundred microlitres of each culture was pipetted onto the agar. The cultures were mixed together on the surface of the agar using a flame sterilized glass spreader. Incubation was for 3 days at 28°C.

After incubation, a sterile inoculating loop was used to remove two loopfuls of cells from the surface of the agar. These cells were resuspended in 500 μl LB in a sterile Eppendorf tube and then heavily streaked out onto two LA plates. These plates contained selective antibiotics to select for the *Agrobacterium* recipient strain containing an introduced plasmid. The selection plates were incubated for two days at 28°C, during which time single colonies of transconjugant *A. tumefaciens* appeared. A number of these colonies were selected for further purification. Selected colonies were streaked out onto antibiotic plates to ensure pure, single colonies. The transconjugant strains were checked for the presence of the vector plasmid following miniprep DNA extraction and Southern blot analysis.

2.2.6 Southern hybridization analysis of *Agrobacterium tumefaciens* transconjugant strains. The presence of pJIT119 DNA in the transconjugant *Agrobacterium* strains C58C1(pGV2260)(pJIT119) and C58C1(pMP90)(pJIT119) was confirmed using Southern hybridization (Southern, 1975; Sambrook *et al.*, 1989). After plasmid DNA was extracted from these two strains and separated by agarose gel electrophoresis, the agarose gels were processed to transfer the DNA to nitrocellulose paper (Sambrook *et al.*, 1989). The hybridization process was carried out according to the procedures outlined in Scott (1988) and Sambrook *et al.* (1989). Autoradiography was used to locate the labelled DNA probes bound to the DNA bands.

Hybridization of the DNA blots was carried out using two [α-^{32}P] labelled DNA probes. The two DNA probes consisted of a T-DNA probe prepared from pGV941 using a *Hind* III/*Eco* RI digest, as well as a GUS gene probe cut from pJIT58/1 with *Bam* HI. These DNA probes were labelled with [α-^{32}P]dCTP to high specific activity by random priming. The reagents were obtained in kit form from Boehringer Mannheim. The [α-^{32}P]dCTP was obtained from Amersham. Approximately 200 ng of each DNA fragment was labelled with [α-^{32}P]dCTP. Using dialysis, unincorporated nucleotides were removed after the preparation of each radioactively labelled probe. Individual nitrocellulose filters (Millipore, 0.22 μm pore size) were floated on distilled water in 9 cm Petri dishes. Small drops (10 μ - 15 μl) of labelled DNA were placed on the filters and dialyzed overnight. Radioactively labelled probes were stored in

lead containers at -20 ° C until required. The radioactively labelled DNA probes were added to the hybridization solution to give approximately 10^6 counts per minute (cpm) per blot (6 cm x 10 cm). Probes were denatured by boiling for five minutes in a fume hood prior to use.

2.2.7 GUS histochemical assay of *Agrobacterium tumefaciens* transconjugant strains. The histochemical assay for ß-glucuronidase (GUS) activity was found to be an effective test to determine the presence of vector plasmids encoding and expressing the (*uidA*) gene in individual bacterial transconjugant strains. This test is only applicable when the *uidA* gene construct possesses an intact bacterial ribosomal binding site and thus is expressed in bacteria (Liu *et al.*, 1992).

The *Agrobacterium* strains which were analysed for ß-glucurinidase activity using the GUS histochemical assay (Jefferson, 1987) were the transconjugant strains C58C1(pGV2260)(pJIT119), C58C1(pMP90)(pJIT119), C58C1(pGV2260)(pBI121), C58C1(pMP90)(pBI121) and LBA4404(pAL4404)(pJIT119). Strains tested as GUS negative controls were C58C1 Rif^r, C58C1(pGV2260), C58C1(pMP90), and the transconjugant strains C58C1(pGV2260(pJIT85)and LBA4404(pAL4404). Individual colonies from each transconjugant strain were resuspended in 100 μl of GUS incubation buffer (Jefferson, 1987) in a plastic microtitre tray. Ten microlitres of GUS substrate 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-gluc) were added. Incubation was at 37 ° C overnight. The presence of an indigo dye in the bacterial suspension confirmed the presence of an active *uidA* gene. This evidence was used to infer the presence of pJIT119 and other *uidA*-containing vector plasmids within bacterial transconjugant strains.

2.3 Results

2.3.1 Restriction enzyme mapping of vector plasmids. From the *Pst* I digest of pJIT119, three DNA bands of approximately 600 bp, 4 kb and 13 kb were obtained. From the *Hind* III digest, the resulting bands were approximately 5 kb and 13 kb in size. From the *Eco* RI digest, the three bands that resulted were 2 kb, 3 kb and 13 kb in size. Uncut pJIT119 ran at a position equivalent to 20 kb. From the *Xba* digest of pJIT58/1, two DNA bands of approximately 2.2 kb and 4.8 kb were obtained. From the *Bam* HI digest, two bands also of 2.2 kb and 4.8 kb resulted. Uncut pJIT58/1 ran at a position equivalent to 5.2 kb. The DNA fragments resulting from the restriction enzyme digests of pJIT119 and pJIT58/1 were, therefore, in the estimated size ranges (Table 2.3)

Tobacco plants transformed with pJIT119 were the main subject of this present study on the genetic engineering of South African tobacco cultivars. For this reason, the restriction enzyme digests of pJIT119 are shown in detail (Fig. 2.1).

2.3.2 *Agrobacterium tumefaciens* transconjugant strains. Each triparental mating resulted in *Agrobacterium* transconjugant strains (Table 2.4). The transconjugant strains C58C1(pMP90)(pJIT119) and C58C1(pGV2260)(pJIT119) were selected for further study.

2.3.3 Southern hybridization analysis of *Agrobacterium tumefaciens* transconjugant strains. Southern blot analysis of *Agrobacterium* transconjugant strains C58C1(pGV2260)(pJIT119) and C58C1(pMP90)(pJIT119) confirmed that the binary vector pJIT119 was present in both of these strains. By probing DNA isolated from these strains with both [α-^{32}P]dCTP labelled T-DNA and *uid*A probes, a 20 kb DNA band was identified. Because of its size and homology with the two probes, this band was likely to be pJIT119. The T-DNA and *uid*A probes did not hybridize with the helper Ti plasmid, pGV2260, confirming that the T-DNA sequences had been deleted from this strain as specified in the literature (Deblaere *et al.*, 1985). The results of Southern blot analysis of C58C1(pGV2260)(pJIT119) with the T-DNA and *uid*A probes are shown in Fig. 2.2 and Fig. 2.3.

Table 2.3. Analysis of DNA fragment sizes following restriction enzyme digestion of pJIT119 and pJIT58/1.

Plasmid	Restriction enzyme	Actual DNA fragment sizes[a]	Estimated DNA fragment sizes [b]
pJIT119	uncut	20.0 kb	17.5 kb
	Pst I	600 bp, 4.0 kb, 13.0 kb	750 bp, 3.45 kb, 13.7kb
	Hind III	5.0 kb, 13.0 kb	3.7 kb, 13.8 kb, 12.3 kb
	Eco RI	2.0 kb, 3.0 kb, 13.0 kb	2.8 kb, 3.55 kb, 12.3 kb
pJIT58/1	uncut	5.1 kb	5.2 kb
	Xba	2.2 kb, 4.8 kb	1.75 kb, 3.45 kb
	Bam HI	2.2 kb, 4.8 kb	1.75 kb, 3.45 kb

[a]Fragment size calculated based on relative mobilities.
[b]Fragment size estimated from key provided with plasmid map and Plant Gene Tool Kit Manual.

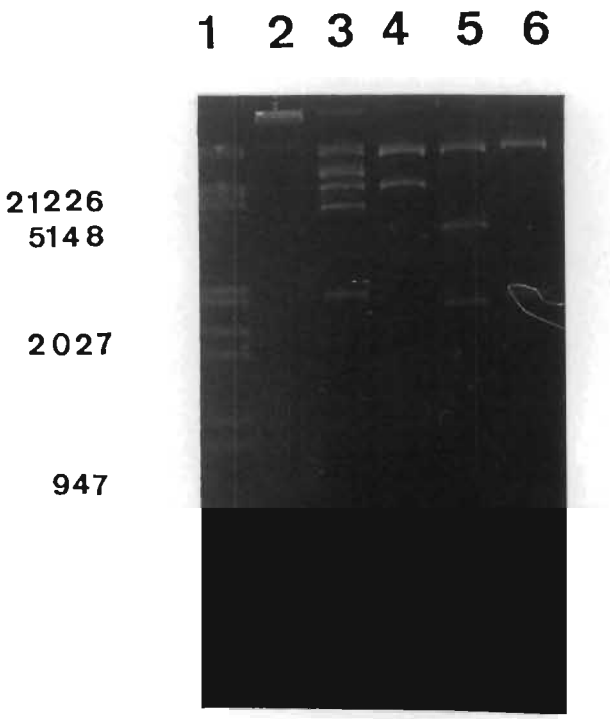


Fig. 2.1. Restriction endonuclease analysis of pJIT119. Lane 1, lambda molecular weight marker III (Boehringer Mannheim); lane 2, pJIT119 uncut; lane 3, pJIT119 cut *Pst* I; lane 4, pJIT119 cut *Hind* III; lane 5, pJIT119 cut *Eco* RI; lane 6, pJIT119 cut *Xba*.

Table 2.4. Transconjugant *Agrobacterium tumefaciens* strains constructed in this study.

STRAINS	ANTIBIOTIC RESITANCE	SOURCE
C581Cl(pGV2260)(pJIT119)	Kan, Carb, Rif	This study
C581Cl(pMP90)(pJIT119)	Kan, Gent, Rif	This study
LBA4404(pAL4404)(pJIT119)	Kan, Rif	This study
C581Cl(pGV2260)(pJIT85)	Kan, Rif	This study
C58Cl (pGV2260)(pBI121)	Kan, Carb, Rif	This study
C58Cl (pMP90)(pBI121)	Kan, Gent, Rif	This study
C58Cl (pGV2260)(pJIT58)	Kan, Carb, Rif	This study

Kan = kanamycin; Carb = carbenicillin; Gent = gentamycin; Rif = rifampicin.

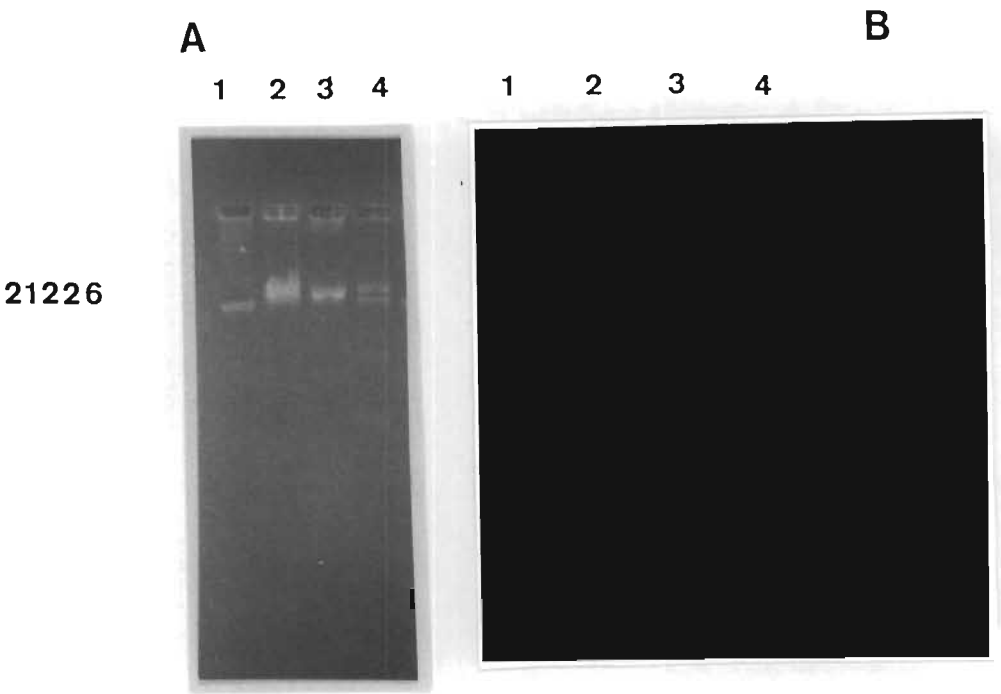


Fig 2.2. Detection of T-DNA in plasmid DNA isolated from *Agrobacterium tumefaciens* transconjugant strain C58C1(pGV2260)(pJIT119). (A) Agarose gel of plasmid DNA. Lane 1, C58C1(pGV2260); lane 2, C58C1(pGV2260)(pJIT119); lane 3, JM101(pJIT119) *E. coli* control; lane 4, lambda molecular weight marker III (Boehringer Mannheim). (B) Autoradiograph of (A) hybridized with [α-^{32}P] dCTP labelled T-DNA probe. Lane 1, C58C1(pGV2260); lane 2, C58C1(pGV2260)(pJIT119); lane 3, JM101(pJIT119) *E. coli* control; lane 4, lambda molecular weight marker III (Boehringer Mannheim).

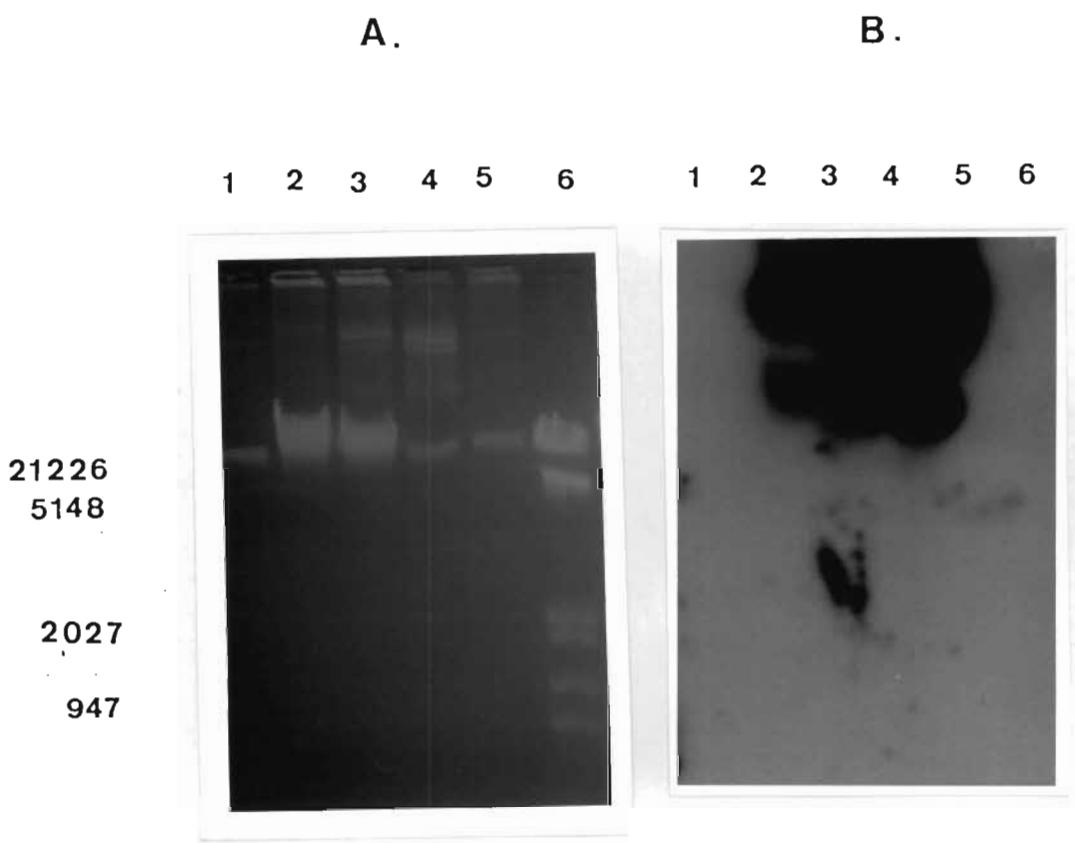


Fig. 2.3. Detection of the *uidA* gene in the plasmid DNA of *Agrobacterium tumefaciens* transconjugant strain C58CI(pGV2260)(pJIT119). (a) Agarose gel of plasmid DNA. Lane 1, C58CI(pGV2260); lane 2, C58CI(pGV2260)(pJIT119); lane 3, JM101(pJIT119) *E. coli* control before cesium chloride purification; lane 4, JM101(pJIT119) *E. coli* control after cesium chloride purification; lane 5, C58CI(pGV2260); lane 6, lambda molecular weight marker III (Boehringer Mannheim). (b) Autoradiograph of (a) hybridized with [α-^{32}p] dCTP labelled *uidA* gene probe. Lane 1, C58CI(pGV2260); lane 2, C58CI(pGV2260); lane 3, JM101(pJIT119) *E. coli* control before final cesium chloride purification; lane 4, JM101(pJIT119) *E. coli* control after final cesium chloride purification; lane 5, C58CI(pGV2260); lane 6, lambda molecular weight marker III (Boehringer Mannheim).

2.3.4 GUS histochemical assay of *Agrobacterium tumefaciens* transconjugant strains. The presence and expression of the *uidA* gene in colonies of the *Agrobacterium* transconjugant strains C58C1(pGV2260)(pJIT119), C58C1(pMP90)(pJIT119), C58C1(pGV2260)(pBI121) and LBA4404(pJIT119) was confirmed using the GUS histochemical assay. Following the assay, the suspensions made from the bacterial colonies accumulated the blue product associated with the activity of ß-glucuronidase (GUS), indicating an active *uidA* gene. Non-transconjugant strains C58C1, C58C1(pGV2260), C58C1(pMP90) or LBA4404 did not accumulate the blue product associated with GUS activity. This was expected as they did not contain plasmids with a *uidA* gene. The GUS histochemical assay, therefore, provided a quick, reliable method to confirm that *Agrobacterium* transconjugant strains were true transconjugants. To be true transconjugants, *A. tumefaciens* colonies must have received a vector plasmid containing an *uidA* gene following triparental mating. The GUS assays of representative transconjugants are presented in Fig. 2.4.

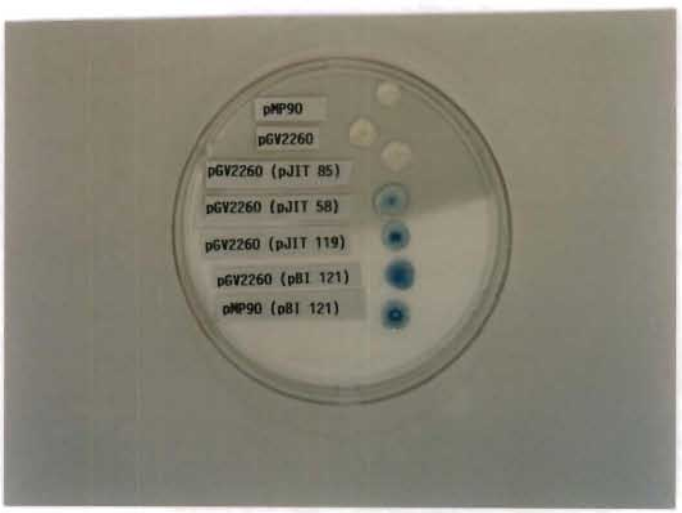


Fig 2.4. The GUS histochemical assay performed on *Agrobacterium tumefaciens* transconjugant strains prepared for this study. This assay was carried out to confirm the presence and expression of the *uidA* gene in these strains. The *Agrobacterium* helper strains C58C1(pGV2260) and C58C1(pMP90) are indicated by "pGV2260" and "pMP90" in the figure. These strains and the *Agrobacterium* transconjugant strain C58C1(pGV2260)(pJIT85) do not possess the *uidA* gene and serve as negative controls for GUS expression. The *Agrobacterium* transconjugant strains C58C1(pGV2260)(pJIT58), C58C1(pGV2260)(pJIT119), C58C1(pGV2260)(pBI121) and C58C1(pMP90)(pJIT119) all demonstrate the blue colour associated with expression of the *uidA* gene.

2.4 Discussion

The triparental mating of each of several *Agrobacterium tumefaciens* helper strains, C58C1(pGV2260), C58C1(pMP90) and LBA4404, with an *E.coli* mobilizing strain HB101(pRK2013) and an *E.coli* donor strain carrying the binary vector pJIT119 or other binary vector plasmids was successful. Many *A. tumefaciens* transconjugant colonies were obtained on selection medium following each triparental mating. One of the transconjugant strains, C58C1(pGV2260)(pJIT119), was used in subsequent studies to produce transgenic tobacco plants expressing a herbicide resistance gene.

Once transconjugant *A. tumefaciens* strains were obtained, no further restriction endonuclease mapping of the vector plasmids was carried out. Draper *et al.* (1988) recommends that this procedure be carried out to verify the structure of the vector plasmid following conjugation, as disabling rearrangements of the T-DNA structure can occur during the conjugation process (Draper *et al.*, 1988). In this study, however, indirect tests were found to be satisfactory in order to confirm that the binary vector, pJIT119, was present in the bacterial transconjugants. These tests also confirmed that T-DNA encoded genes were functional in these strains. The tests included confirming the ability of the *Agrobacterium* transconjugant strains to grow on a full antibiotic complement, as well as confirming the activity of the *uidA* gene through the GUS histochemical assay.

Southern blot analysis also confirmed the presence of T-DNA and the *uidA* gene in transconjugant strains. Southern analysis added to the evidence that pJIT119 was fully functional in the transconjugant strains. The plasmid was found to have undergone no visible size changes in the transconjugant strains. The presence of plasmid DNA of the expected size for pJIT119 (± 20 kb) within the transconjugant strain C58C1(pGV2260)(pJIT119) was confirmed. Thus, the anticipated problem of disabling plasmid DNA rearrangements occurring during the bacterial conjugation procedure did not arise.

Thus the goal of this study to produce *A. tumefaciens* transconjugant strains

carrying the binary vector pJIT119 for the genetic engineering of South African tobacco cultivars was achieved.

Chapter 3

Transformation of South African commercial tobacco cultivars.

Chapter 3

Transformation of South African commercial tobacco cultivars.

3.0 Summary

The South African tobacco cultivars J6, TL33 and 20/19, as well as the cultivar Samsun, proved to be amenable to *in vitro* manipulation and to genetic engineering using *Agrobacterium*-mediated transformation. Tobacco mesophyll protoplasts were isolated and transformed using direct DNA transformation. Tobacco single cell cultures were also prepared and co-cultivated with *Agrobacterium*. Other methods of introducing foreign DNA into plants were evaluated in this section. Asulam (Maybaker) resistant transgenic plants of the South African commercial tobacco cultivars were produced using an *Agrobacterium*-mediated leaf disc transformation method. All South African cultivars proved amenable to leaf disk transformation.

3.1 Introduction

There are many approaches to the genetic engineering of tobacco, including the use of *Agrobacterium* and direct DNA transfer. In this section, several of these approaches are evaluated. Due to its simplicity and efficiency, the leaf-disc transformation technique using *Agrobacterium tumefaciens* was found to be the method of choice for tobacco (Herrera-Estrella and Simpson, 1988). There are two features which make the leaf disc transformation method suitable for tobacco. Firstly, regeneration of shoots from the cut leaf edge occurs readily. Secondly, tobacco leaf segments do not contain quiescent buds or preformed organ primordia. Therefore, shoot regeneration from cut leaf edges originates from single undifferentiated cells. When transformed, each of these cells can lead to a single transgenic plant, minimizing chimaerism (Draper *et al.*, 1988). Chimaeric transformants contain transformed as well as untransformed sectors. Chimaerism is an undesirable characteristic as chimaerically transformed plants sometimes fail to produce transgenic progeny (Horsch *et al.*, 1985).

The *Agrobacterium*-mediated leaf-disc transformation technique involves the infection of the cut edges of leaves with an *Agrobacterium* strain which contains a Ti plasmid based gene expression vector. After several days of co-cultivation, the leaf pieces are placed on a suitable regeneration medium. This medium must also contain an antibiotic or a herbicide to select for transformed tissue and antibiotics to eliminate the *Agrobacterium* inoculum from the plant material in culture. Small shoots develop along the cut edges of the leaf discs and regenerate into transgenic plantlets after continued culture on regeneration medium. When the regeneration medium contains an antibiotic to select for transformed plant cells, the shoots developing along the cut leaf edges are likely to be transgenic. The most commonly used antibiotic for transgenic plant selection is kanamycin. The *nptII* gene confers resistance to kanamycin in transgenic plants (Horsch *et al.*, 1985; Horsch and Klee, 1986; Horsch *et al.*, 1986; An *et al.*, 1986).

To complete the production of transgenic tobacco plants from an *Agrobacterium*-mediated leaf disc transformation experiment, small shoots obtained from explants

cultured on selective medium are excised from the leaf pieces. These shoots are transferred to a tissue culture medium which allows root formation. The inclusion of the antibiotic kanamycin in the rooting medium selects for those shoots which are transgenic. Plantlets which are not transgenic fail to root. Any shoots which fail to root or are bleached are discarded at this stage. When roots have formed on the transgenic plantlets, the plantlets are removed from the *in vitro* culture medium, placed in sterile soil or vermiculite and grown under greenhouse conditions until flowering. The progeny can be tested for foreign gene inheritance (Herrera-Estrella and Simpson, 1988).

The *Agrobacterium*-mediated leaf disc method does not require a high level of tissue culture expertise and can be applied to many different plant species. The protocols are readily available and can be applied without the need for extensive optimization. The technique is easily modified for use with explants other than leaves, depending upon which part of the plant is most responsive to regeneration. The list of plant species transformed using the leaf disc transformation method is ever-increasing indicating the wide application of this technique. Plants transformed with *Agrobacterium*-mediated leaf disc transformation include liquorice, strawberry, clover, sunflower, alfalfa, maize, tomato, potato, lettuce, celery, poplar, flax and cotton (Horsch *et al.*, 1988; Jones, 1992).

Tobacco single cells are also readily transformed using *Agrobacterium* strains containing vector plasmids. This approach can be useful to create many hundreds of individual transformed tobacco plantlets from a single transformation experiment (Draper *et al.*, 1988). Tobacco regenerates well from callus and single cells cultured in liquid medium. The growth characteristics of the tobacco cells in liquid culture should be established early on so that the *Agrobacterium* inoculum can be added when the cells are competent for transformation ie. during cell division (Draper *et al.*, 1988). The disadvantage of using cell or callus cultures is that genetic aberrations accumulate during prolonged culture. A greater genetic stability is found in situations where organogenesis is induced directly from explant material.

Many novel transformation methods which do not utilize the natural gene transferring

ability of *Agrobacterium tumefaciens* have been developed for the transformation of tobacco. These methods, known as direct DNA transfer methods, generally introduce "naked" DNA into tobacco protoplasts or apical meristems. Protoplasts are plant cells from which the cell walls have been removed by enzymes and into which DNA can be introduced by methods such as electroporation or chemically via the use of PEG. The apical meristem differentiates into the mature plant during normal development and foreign genes introduced into the apical meristem at an early stage will later be found throughout the mature plant. A variety of direct DNA approaches exist, using either chemical treatment (polyethylene glycol), an electrical method (electroporation) or a physical treatment (the particle gun) (Potrykus, 1991). Direct DNA transfer, as well as *Agrobacterium*-mediated transformation, both have inherent limitations and each can only be applied to transfer genes to plants under certain conditions. A wide variety of methods will possibly always be required for future gene transfer projects (Potrykus, 1991). At this stage, the particle gun method of direct DNA transfer is probably the most universal method for the transformation of plants (Yamashita *et al.*, 1991; Russell *et al.*, 1992; Haigo *et al.*, 1991; Stomp *et al.*, 1991; Bidney *et al.*,1992).

Other DNA transfer methods have been devised, but have not conclusively resulted in stably transformed plants. These methods include imbibition of dry seeds in a solution of DNA (Töpfer *et al.*, 1989), pollen transformation (Fennell and Hauptmann, 1992; Van der Leede-Plegt *et al.*, 1992) and the use of cut pollen tubes to transfer DNA to the zygote (Luo *et al.*, 1988). Liposome fusion (Caboche, 1990) is also a well established technique for producing transgenic plants, but offers no advantages over simpler methods of gene transfer (Potrykus, 1991). Ultrasonication of leaf tissue can also sometimes be used to deliver naked DNA to plant tissues (Zhang *et al.*, 1991; Joersbo and Brunstedt, 1992). Gene transfer systems based on viral vectors have also been extensively studied, but none have yet yielded a general gene transfer system for plants (Herrera-Estrella and Simpson, 1988).

In general, certain biological parameters affect the transfer to and expression of foreign genes in plant cells. These parameters exist regardless of the means used to transfer foreign genes into plant cells (Potrykus, 1991). Plant tissue is a mixed

population of cells at different stages of development. Only a very small proportion of cells in an explant is competent for transformation and regeneration at any one time. Under normal circumstances, transgenic plants can be regenerated only from cells competent for both transformation and regeneration. The most effective trigger for shifting dicotyledonous plant cells potentially competent for regeneration into the fully competent state is mechanical wounding. The wound response in dicotyledonous plants is probably also the biological basis for cell proliferation and shoot regeneration from somatic cells (Potrykus, 1991). For this reason, explants are cut or wounded in other ways in *Agrobacterium*-mediated transformation procedures. *Agrobacterium tumefaciens* also relies on the plant wound response for the induction of the T-DNA transfer process. The economically important graminaceous plant species and grain legumes (eg. soya) have only a rudimentary wound response and it is therefore difficult to obtain transformation and regeneration simultaneously (Potrykus, 1991). Fortunately, tobacco has an excellent wound response and most cells at the wound site can become competent both for transformation and regeneration simultaneously, making tobacco an ideal plant for the production of transgenic plants.

Using the *Agrobacterium*-mediated leaf disc transformation method, herbicide (asulam) resistant transgenic tobacco plants of South African tobacco cultivars were obtained. At the time of this study, no South African commercial tobacco cultivars had been transformed. Also, other methods of introducing foreign DNA into plants were evaluated in this section.

3.2 Materials and Methods

3.2.1 Source of tobacco seed. South African commercial tobacco (*Nicotiana tabacum* L.) cultivars TL33, 20/19 and J6 were used in the study. Seeds were obtained through the courtesy of Dr Ben Strydom, director of the Tobacco and Cotton Research Institute, Rustenburg, South Africa. Seeds of cv. Samson were obtained from Dr Gerard Pieterson, Virology Institute, Agricultural Research Council Plant Protection Research Institute, Pretoria. Plants of cv. White Burley were obtained from Dr Arlene Bayley, Department of Botany, University of Natal, Pietermaritzburg.

Tobacco plants were grown from seed and cultivated under greenhouse conditions (temperature range 15 °C - 32 °C during summer) until the six leaf stage. Following this, the plants were relocated to an indoor plant-room with a natural light cycle supplemented with continuous fluorescent lighting (180 $\mu Em^{-2}s^{-1}$) where they were grown to maturity. Flower buds were removed to keep the plants compact and prevent senescence. Mite infestations of the plant room were prevented through the regular use of Vapona strips (Shell S.A. (Pty) Ltd.) containing dichlorvos.

3.2.2 Culture media for the *in vitro* culture of South African tobacco cultivars. To establish the *in vitro* shoot and callus cultures of South African tobacco cultivars, fully expanded tobacco leaves selected from plant-room grown tobacco plants were used to provide explant material. The leaves were washed under running water to remove dust and soil particles before surface sterilization. Surface sterilization was carried out by immersing the leaves in a 33% solution of domestic bleach (1.15% final sodium hypochlorite concentration) containing 0.01% Tween 20 for twenty minutes. The leaves were then rinsed twice in sterile water to remove all traces of the bleach solution. To prepare the leaf material for shoot or callus induction *in vitro*, the leaf margins, midrib and any bleached or damaged areas were removed from the surface sterilized leaves. The leaves were cut into 1 cm^2 squares and placed onto shoot induction medium or callus induction medium.

The MS basal medium of Murashige and Skoog (1962) containing 30g/l sucrose

and 0.8% agar was used for all tissue culture manipulation of tobacco. Shoot induction medium was prepared from MS basal medium by the addition of 1.0 mg/l benzylaminopurine (BA) and 0.1 mg/l indole-3-acetic acid (IAA) (Draper *et al.*, 1988). This medium caused adventitious shoot development along the cut edges of tobacco leaves *in vitro*. To select for transformed tobacco shoots in a leaf disc transformation experiment, kanamycin (Sigma) (100 mg/l) was included in the medium. To eliminate *Agrobacterium* from the tobacco culture following a leaf disc experiment, the antibiotic cefotaxime (Roussell) was added at a concentration of 1.0 g/l (Herrera-Estrella and Simpson, 1988).

Tobacco callus induction medium was prepared from MS basal medium (Murashige and Skoog, 1962) by the addition of 0.5 mg/l BA and 2.0 mg/l 1-naphthaleneacetic acid (NAA) (An, 1985; Herrera-Estrella and Simpson, 1988). To induce callus, surface sterilized tobacco leaf pieces were placed on this medium and incubated in the dark at 25°C for six to eight weeks. Several subcultures during this period facilitated the rapid appearance of callus. To perform a callus induction assay on transgenic leaf material, kanamycin (100 mg/l) was included in the medium. The callus induction assay was a useful aid in determining whether or not tobacco shoots obtained from a leaf disc transformation experiment were transformed. Transformed tobacco tissue containing an *nptII* gene encoding kanamycin resistance formed callus on this medium, while untransformed tissue did not (Reynaerts *et al.*, 1988).

Rooting medium was also prepared from MS basal medium. No hormones are included in this medium. Rooting medium containing kanamycin (100 mg/l) was used to select for transgenic plantlets. Transgenic tobacco plantlets expressing a *nptII* gene produced roots on this medium, while untransformed plantlets did not (Reynaerts *et al.*, 1988).

3.2.3 Minimum inhibitory concentrations of the herbicide and the antibiotics required in tobacco transformation. To set up a system to genetically engineering South African tobacco cultivars, the levels of kanamycin monosulfate (Sigma), asulam (Maybaker) and cefotaxime (Roussel) toxic to untransformed tobacco leaves

inhibitory concentration (MIC) of kanamycin which inhibited adventitious shoot initiation and callus development from tobacco leaf pieces *in vitro* was determined. Untransformed tobacco leaf pieces were placed on shoot inducing MS (Murashige and Skoog, 1962) medium or callus inducing MS media containing a range of kanamycin concentrations (0 mg/l, 20 mg/l, 50 mg/l, 100 mg/l and 200 mg/l). The concentration of kanamycin causing noticeable bleaching of the leaf pieces and inhibition of adventitious shoot formation on shoot inducing MS medium was recorded. The minimum level of kanamycin inhibiting callus formation from untransformed leaf pieces on callus inducing medium was also recorded. These kanamycin concentrations formed the basis for the selection of transgenic shoots during a leaf disc transformation experiment. They were also used to screen transformed shoots produced during a leaf disc transformation experiment.

For each tobacco cultivar, the sensitivity of leaf explants to the herbicide asulam (Maybaker) was also determined. The commercial asulam preparation contained 400g/l of the active ingredient methyl (4-aminobenzenesulphonyl) carbamate. Shoot inducing MS medium containing different concentrations of asulam (0 mg/l, 20 mg/l, 50 mg/l, 100 mg/l and 200 mg/l) was prepared. Surface sterilized untransformed tobacco leaves were cut into 1 cm^2 pieces and cultured on the asulam containing media for four weeks in the light (62.5 $\mu Em^{-2}s^{-1}$). The concentration of asulam which caused a noticeable inhibition of shoot formation was recorded. This concentration was subsequently used to assay transgenic tobacco tissue for expression of the asulam resistance gene (*sul* I).

Cefotaxime was used in the *Agrobacterium*-mediated transformation experiments to eliminate the *Agrobacterium* from the tissue culture once transformation was complete. Claforan (cefotaxime, sodium salt) was purchased from Roussell Laboratories (Pty) Ltd. Shoot inducing MS medium containing different concentrations of cefotaxime (0 mg/l, 250 mg/l, 500 mg/l and 1000 mg/l) was also prepared. Surface sterilized untransformed tobacco leaf pieces were cultured on this medium for four weeks in the light. The concentration of cefotaxime which allowed the production of adventitious shoots from tobacco leaves was recorded. To test the effectiveness of cefotaxime in controlling *Agrobacterium tumefaciens* in culture,

effectiveness of cefotaxime in controlling *Agrobacterium tumefaciens* in culture, MS/LA plates were prepared. These plates were made with a 1:1 ratio of MS (Murashige and Skoog, 1962) basal medium (containing 12 % agar) and Luria agar (LA). The cefotaxime concentrations used in this medium were the same as those included in the shoot inducing medium. The *Agrobacterium tumefaciens* strain C58C1(pGV2260)(pJIT119) was streaked out on these plates and incubated at 28 °C to determine the level of cefotaxime most effective in controlling *Agrobacterium* in culture.

3.2.4 Isolation and culture of tobacco mesophyll protoplasts. The isolation of tobacco mesophyll protoplasts for use in direct gene transfer experiments was carried out according to Power *et al.* (1984); Power and Chapman (1986) and A. Bayley (pers. comm.). Mannitol was used as the osmoticum, while a sucrose solution was used to separate viable protoplasts from cell debris using centrifugation and flotation. A protoplast washing solution, CPW salts (Power *et al.*, 1984; Power *et al.*, 1985), was used during the initial stages of protoplast isolation, including the enzyme isolation from peeled leaves and in the washing stages of protoplast preparation The enzyme mixture to release the protoplasts from peeled tobacco leaves contained 0.5% Onozuka R10 (cellulase) and 0.1% Macerozyme (pectinase), made up in CPW salts containing 13% mannitol (CPW13M) (Power *et al.*, 1984).

To initiate the protoplast isolation procedure, the lower epidermis of large, surface sterilized tobacco leaves of cv. White Burley and 20/19 were removed by careful peeling. The peeled leaf pieces were cut into small (2 - 3 cm^2) squares and transferred to CPW13M (Power *et al.*, 1984) solution for plasmolysis. After an hour, the CPW13M solution was removed and replaced with the enzyme solution. The leaf pieces were incubated in the enzyme solution 16 hours at 27 °C, in the dark. After incubation, the enzyme mixture was replaced with CPW13M solution. The leaf pieces were swirled gently in this medium to release the protoplasts. The CPW13M medium containing the released protoplasts was transferred to a sterile 15 ml centrifuge tube and the protoplast suspension centrifuged for 10 minutes at 50 x g. The pellet containing both protoplasts and plant cell debris was resuspended in 10 ml of CPW21S which is CPW salts containing 21% sucrose (Power *et al.*, 1984). The

protoplasts and other debris were centrifuged for 10 minutes at 50 x g. The viable protoplasts floated on the surface of this medium and were collected into a sterile centrifuge tube. The protoplasts were finally resuspended in 10ml of protoplast culture medium, MSP_19M (Power *et al.*, 1984). Protoplast enumeration was carried out at this stage using a haemocytometer. The cell count was adjusted to 1×10^5 protoplasts per ml with MSP_19M (Power *et al.*, 1984).

Cell viability was established using the flourescein diacetate (FDA) retention method (Power *et al.*, 1984). A stock solution of FDA (5 mg/ml in acetone) was prepared and stored at 0 °C in the dark. One drop was mixed with 10 ml CPW13M medium. Equal volumes of this solution were mixed with a dense protoplast suspension in MSP_19M culture medium. After five minutes, the fluorescence of the protoplasts was examined using fluorescence microscopy.

To plate out the protoplasts for regeneration, the protoplasts were embedded in agar. To achieve this, 3 ml of a protoplast suspension in MSP19M was added to 3 ml of lukewarm $MSP_19M0.6$ agar medium (Power *et al.*, 1984). The mixture was poured into a small (5 cm diameter) Petri dish, and allowed to set. The solidified agar containing the protoplasts was then divided into quarters and each quarter transferred to an empty 9 cm Petri dish. Initially, 10 ml of MSP_19M was added to each quadrant in the Petri dishes, and at weekly intervals, 2.5 ml of MSP_19M was removed from the Petri dish and replaced with 2.5 ml of MPS1 (Power *et al.*, 1984) to replenish the medium. This method also allowed the slow dilution of the osmoticum. Culture of the protoplasts was carried out under a high light intensity (62.5 $\mu Em^{-2}s^{-1}$) at 26 °C. After four weeks of culture, microcalli had developed. These were removed from the agar and transferred to shoot inducing medium for further regeneration.

3.2.5 Direct DNA transfer to tobacco mesophyll protoplasts. Both electroporation and polyethylene glycol (PEG) treatments were carried out, followed by an assay for transient GUS activity and a DNA-dot blot assay, to determine transformation effectiveness.

3.2.5.1 Electroporation of tobacco mesophyll protoplasts. A Hoefer ProGenetor PG200 electroporation apparatus fitted with a parallel plate electrode was used to electroporate tobacco mesophyll protoplasts. Electroporation conditions which allowed at least 50% of the protoplasts to survive the treatment were determined experimentally by varying voltage, capacitance and pulse duration during electroporation. The buffer (non-conductive electroporation Buffer I, Hoefer Progenetor manual) used for the electroporation was 109.3 g mannitol in 1 l distilled water. The electroporation buffer was adjusted to pH 5.5 with 5 M NaOH. The electroporation procedure was performed in sterile Nunc four-well trays. Supercoiled DNA, extracted from *E.coli* JM83(pJIT58/1) and purified according to Birnboim and Doly (1979) was used for the electroporation procedure. This DNA was added to a concentration of 50 μg/0.35 ml of electroporation buffer in each Nunc well. The protoplasts were introduced into the wells by adding a 150 μl aliquot of protoplast suspension in Buffer I. The final protoplast density was 7 x 10^5 cells per ml, in a final volume of 0.5 ml. After the electrical pulse was given, the electroporated protoplasts were kept stationary for 10 minutes to allow the membrane pores to close. After this rest period, 1 ml MSP,9M culture medium was added very slowly. The electroporated protoplast suspension was then gently taken up in a wide-mouthed pipette and further diluted to a concentration of 1 x 10^5 cells/ml. After 40 hours incubation at room temperature in MSP,9M, aliquots were removed and assayed for GUS activity using the histochemical assay (Jefferson, 1987). DNA was also extracted from the electroporated protoplasts (Draper *et al.*, 1988).

3.2.5.2 Polyethylene glycol (PEG) transformation of tobacco mesophyll protoplasts. The polyethylene glycol (PEG) method of Draper *et al* (1988) was used to transfer plasmid DNA into protoplasts. The effect of different concentrations of PEG 6000 (5% - 30%) on protoplast survival and DNA uptake was investigated. The protoplasts were prepared in MSP,9M (Power *et al.*,1984). To transform these protoplasts, the MSP,9M medium was removed from the protoplasts, leaving only a thin film covering the pellet. To the pellet, 20 μl of plasmid DNA solution (1 μg/μl of pJIT58/1) was added. Next, 2 ml of either a 5%, 10%, 20% or 30% PEG solution was added slowly to the protoplasts. One the protoplasts were fully mixed with the different concentrations of PEG, they were incubated at 28°C for 30 minutes with

occasional gentle swirling. After 30 minutes, the incubation mixture was diluted stepwise by slowly adding 10 ml of MSP,9M. The protoplasts were pelleted by centrifugation at 50 x g. The pellet was resuspended in 1 ml of MSP,9M.

3.2.5.3 Extraction of tobacco mesophyll protoplast DNA and DNA dot-blot analysis. A DNA dot blot method (Draper *et al.*, 1988) was used to determine plasmid DNA uptake by protoplasts following electroporation and PEG treatments. To inactivate plasmid DNA adhering to the surface of the protoplasts or contained in the protoplast buffer solution, 10 μl of a DNase solution (4 mg/ml in 20 mM $MgCl_2$) was added to the protoplasts. Incubation was at room temperature for 30 minutes (Draper *et al.*, 1988).

Following the DNAase treatment, the total protoplast DNA was extracted after either the electroporation or the PEG treatments. The protoplasts were lysed in a 3X CTAB extraction buffer (Draper *et al.*, 1988) and the DNA precipitated in CTAB precipitation buffer (Draper *et al.*, 1988). The DNA extracted from the protoplasts was dot-blotted onto nitrocellulose paper, baked at 80°C and probed with a DIG-labelled (Boehringer Mannheim) pJIT119 whole plasmid probe. This plasmid has many regions of homology with pJIT58/1, namely the *uidA* gene and *nptII* gene. This probe was DIG labelled according to the manufacturers instructions.

3.2.5.4 Assay of transient GUS gene activity in tobacco mesophyll protoplasts. The histochemical assay method of Jefferson (1987) was performed on aliquots of electroporated or PEG-treated protoplasts, as well as on control untreated protoplasts. The assay was performed within 48 hours following electroporation or PEG treatments.

3.2.6 Initiation of tobacco single cell cultures. To initiate tobacco single cell culture, three to four large pieces of friable callus (1.0 - 1.5 cm diameter) were added to 200 ml liquid cell cultures medium in a 1 l flask. Single cell culture medium was a MS basal medium (Murashige and Skoog, 1962) containing 0.5 mg/l BA and 2.0 mg/l NAA. The callus/cell culture was shaken at 100 rpm in the dark at 25°C. A large initial inoculum was required to provide a high cell density necessary for

optimal growth of the cells (Dixon, 1985). To establish a homogeneous cell culture, the initial subculturing of the cell cultures included a step allowing the cells and cell clumps to settle out for thirty minutes. Only the cells from the upper half of the culture were transferred to new medium in a 1:1 ratio (Dixon, 1985). This was repeated weekly as required until a fine cell suspension resulted. Thereafter, the cell cultures were routinely subcultured every seven days by diluting the cells 1:1 with fresh medium (Dixon, 1985).

The regeneration of untransformed tobacco cells from a liquid cell culture was performed by allowing the cells to settle for 45 minutes and replacing half of the liquid MS culture medium with either fresh shoot inducing or callus inducing MS medium containing 1.6% agar at 45 °C. The agar and cells were gently mixed and poured into Petri dishes. Incubation was in the light ($87.5\mu Em^{-2}.s^{-1}$) at 26 °C (Malmberg *et al.*, 1984). Microcalli developed after six weeks culture in callus inducing MS medium, and shoots resulted after eight to ten weeks of culture in shoot inducing MS medium.

3.2.7 *Agrobacterium*-mediated transformation of tobacco single cell cultures. To transform tobacco cell cultures with *Agrobacterium*, 0.1 ml of an overnight broth culture of C58C1(pGV2260)(pJIT119) was added to 50 ml of a tobacco cell suspension culture. The tobacco cell culture had been cultured for four days in liquid cell culture MS (Murashige and Skoog, 1962) medium. Co-cultivation was carried out for two days at 26 °C. To eliminate the *Agrobacterium* inoculum from the tobacco cell culture, the culture medium was replaced every second day with fresh liquid cell culture medium containing kanamycin (100 mg/l) and cefotaxime (500 mg/l). This was repeated four times. Finally, the transformed tobacco cells were mixed with an equal volume of double strength shoot induction MS medium containing cefotaxime (500 mg/l), kanamycin (100 mg/l) and 1.6% agar. The cells and agar were gently mixed together and poured into Petri dishes. Incubation was in the light ($87.5\ \mu Em^{-2}s^{-1}$) at 26 °C until microcalli appeared (Malmberg *et al.*, 1984).

3.2.8 *Agrobacterium*-mediated transformation of tobacco leaf discs. *Agrobacterium*-mediated tobacco leaf disc transformation was carried out according

to published literature (Draper *et al.*, 1988; Horsch *et al.*, 1988; Herrera-Estrella and Simpson 1988). To initiate the procedure, large, newly expanded tobacco leaves of cv. TL33, A4, J6, 20/19 and Samsun were surface sterilized, rinsed and cut into 1 cm^2 squares. The leaf pieces were precultured on shoot inducing MS (Murashige and Skoog, 1962) medium without antibiotics at a low light intensity (2.5 $\mu Em^{-2}s^{-1}$) for two days at 26°C to allow the wound response to develop. After the preculture period on shoot inducing MS medium, the leaf squares were inoculated with a 1/50 dilution of an overnight culture of *Agrobacterium* made up in sterile distilled water. Care was taken not to excessively wet the leaves with the diluted culture as the leaf pieces quickly become waterlogged. The inoculated leaves were cocultivated with the *Agrobacterium* inoculum on MS shoot inducing MS medium for two days at 26°C, at the same low light-intensity. After this, the inoculated leaf pieces were transferred to selection medium. The selection medium was shoot inducing MS medium containing kanamycin (100 mg/l) and cefotaxime (500 mg/l. Incubation of the leaf pieces on shoot inducing medium was at a high light intensity (62.5 $\mu Em^{-2}s^{-1}$) at 26°C. Control experiments with uninoculated tobacco leaves were also set up. Uninoculated tobacco leaves were placed on shoot inducing medium containing both kanamycin and cefotaxime and on shoot inducing medium containing cefotaxime only. The production of shoots from uninoculated tobacco leaves on these media was compared with that obtained from inoculated tobacco leaves.

The leaf discs were observed periodically for the formation of adventitious shoots from the cut edges of the leaves. Shoots formed after ten to twelve weeks. When shoots were large enough to be transferred (0.5 - 1.0 cm), they were excised from the leaf edges. After careful trimming, the shoots were transferred to root inducing MS medium containing kanamycin (100 mg/l) to allow rooting to proceed. Cefotaxime (1000 mg/l) was included at this stage to prevent *Agrobacterium* regrowth. Root inducing medium is MS medium without phytohormones. Roots appeared on transformed shoots within twelve days. No roots developed on untransformed shoot material cultured on rooting medium containing kanamycin.

Individual transgenic plantlets were multiplied through the culture of nodal segments. The leaves were removed from *in vitro* plantlets and the main stem divided into

nodal segments. The cut end of each nodal segment was placed in rooting medium containing kanamycin (100 mg/l) for rooting. After six weeks, both roots and new leaves had developed.

3.2.8.1 Hardening off of *in vitro* grown transgenic tobacco plants. Rooted tobacco plantlets cultured *in vitro* were hardened off through a gradual acclimation process. The plants were removed from the agar without breaking the roots. Adhering agar was washed from the roots with a 1% (w/v) solution of the fungicide, Benlate (Du Pont). Plastic flower pots were sprayed with 70% ethanol, dried and filled with autoclaved commercial potting soil. The washed plants were carefully planted in the sterile potting soil, and the soil watered thoroughly. The plants in their pots were placed in clear plastic bags which were then sealed to prevent desiccation. After the first week, the corners were cut off each bag to decrease the humidity slowly. After a second week, the entire top of the bag was cut open. The plants were well watered at this stage. A week later, the plastic bags were removed from each pot as the plants were fully hardened off at this stage (Herrera-Estrella and Simpson, 1988; A. Bayley, pers. comm.). Hardened-off transgenic plants flowered and set seed after four months. To prevent the escape of transgenic tobacco pollen or seed into the greenhouse or the environment, the flowers were covered with small cellophane oven bags. A corner of each bag was removed to allow ventilation. The seed was collected from the seed pods which had been allowed to dry *in situ*. Seed was stored in glass bottles at 4°C.

3.2.8.2 DNA dot-blot analysis of DNA from transgenic tobacco shoots. Genomic DNA was extracted from fresh transgenic tobacco leaf material using the method of Dellaporta *et al.* (1985). In this method, protein and polysaccharide contaminants were removed by complexing with potassium dodecyl acetate and precipitated from the plant extract. Following centrifugation, the nucleic acids remain in solution (Draper and Scott, 1988). To initiate the DNA extraction process, one gram of young leaf material was frozen in liquid nitrogen and transferred to a prechilled (-20°C) mortar for grinding. A small amount of alumina was used to facilitate the breakdown of the frozen tissue. The resulting powder, which was dry and pale green in colour, was transferred to a 38 ml polyallomer centrifuge tube and

15 ml DNA extraction buffer (Dellaporta *et al.*, 1985) added. The slurry was stirred with a plastic disposable pipette. When a fine suspension was achieved, 2 ml of 10% SDS was added, and after thorough mixing, the tubes containing the plant material were incubated in a water bath at 65°C for 12 minutes, with occasional inversion.

After incubation, 5 ml of 5 M potassium acetate was added and the plant extract was mixed by repeated inversion, ensuring that the heavy salt solution did not remain in a layer at the bottom of the tube. The tubes were incubated on ice for 30 minutes. The protein/SDS complex was pelleted by centrifugation at 12 000 rpm for 30 minutes (4°C), after which the supernatant was poured through a plastic funnel packed with polypropylene wool (wadding for stuffing pillows), into a clean centrifuge tube. To this filtrate was added 15 ml of cold (-20°C) isopropanol. The DNA and isopropanol was mixed carefully by inversion and incubated at -20°C for 30 minutes. The nucleic acids were pelleted by centrifugation at 8000 rpm for 20 minutes. The supernatant was poured off and the pellet partially dried by inverting the tubes onto absorbent paper tissues.

The DNA pellet was gently redissolved in 700 μl of TE buffer (Sambrook *et al.*, 1989), and transferred to a sterile Eppendorf tube. The DNA was centrifuged in a microfuge for 10 seconds to remove insoluble contaminants. The supernatant was transferred to a new Eppendorf tube and 75 μl of 3 M sodium acetate was added and mixed in well. Ice cold isopropanol (500 μl) was added to the supernatant, and the mixture inverted repeatedly until a fibrous nucleic acid precipitate was formed. The DNA pellet was collected following centrifugation for 30 seconds. The supernatant was carefully poured off and discarded. The DNA was finally dissolved in 150 μl of TE.

Methoxyethanol partitioning (Draper and Scott, 1988) was used to remove polysaccharide material from the DNA preparation. To 150 μl of final DNA preparation, 150 μl of 2.5 M K_2PO_4 was added, followed by 150 μl of 2-methoxyethanol. The DNA was mixed well and centrifuged at 12 000 rpm for 10 minutes. The aqueous phase was transferred to a new tube and 2.5 volumes of ice-

cold ethanol was added. The DNA was centrifuged at 12 000 rpm for 10 minutes. After centrifugation, the precipitated DNA pellet was washed in 70% ethanol, dried, and resuspended in 150 μl TE. The DNA concentration was determined by electrophoresis and comparison to lambda DNA of a known concentration.

DNA extracted from each transgenic tobacco plant, as well as from untransformed tobacco (cv. TL33) was analyzed using a dot blot method (Draper *et al.*, 1988). Aliquots of DNA were heated for five minutes in a boiling water bath, and then chilled on ice for two minutes. Each sample was spotted onto nitrocellulose paper in 10 μl amounts, allowing the samples to dry between applications, until 10 μg of DNA of each sample had been loaded. The nitrocellulose membrane was placed onto absorbent filter paper soaked in denaturing solution (Sambrook *et al.*, 1989) and left for one minute. The nitrocellulose membrane was then transferred onto filter paper soaked with neutralizing solution (Sambrook *et al.*, 1989) for one minute. Excess moisture was removed from the nitrocellulose membrane by blotting with filter paper. The membrane was finally allowed to air dry. The membrane was baked at 80 °C for two hours to fix the DNA to the membrane. Finally, the membrane was probed with a radioactively labelled T-DNA probe using the standard Southern blot method (Sambrook *et al.*, 1989). The probe was an [α-^{32}p] radioactively labelled 2.7 kb T-DNA fragment prepared by digesting pGV941 with *Eco* RI and *Hind* III. Labelling of the probe was carried out using a random priming kit (Boehringer Mannheim). The hybridization was detected using autoradiography.

3.3 Results.

3.3.1 The *in vitro* culture of South African cultivars of tobacco. The South African tobacco cultivars listed as TL33, J6, and 20/19, as well as cv. Samsun responded well to *in vitro* manipulation, performing exactly as expected from the methods published in the scientific literature. No cultivar variation in the response of TL33, J6 and 20/19 to *in vitro* culture was observed. No further optimization of methods was required.

3.3.2 Minimum inhibitory concentrations of antibiotics and herbicides required in tobacco transformation. Adventitious shoot development from the cut edges of untransformed tobacco leaf explants from South African cultivars TL33, J6, A4 and 20/19, as well as from the cultivar Samsun, was completely inhibited on shoot inducing medium containing a minimum of 100 mg/l kanamycin (Table 3.1). There was no cultivar variation in the response to kanamycin. As well as inhibition of shoot development, untransformed leaf explants became bleached after prolonged exposure to kanamycin at this concentration and failed to produce shoots on shoot inducing medium (Fig. 3.1). Some shoot development occurred on medium containing 50 mg/l of kanamycin. The concentration of kanamycin at which the efficient selection of transgenic shoots containing the *nptII* gene could be accomplished was 100 mg/l of kanamycin. At this concentration, no adventitious shoots were produced from untransformed leaf discs on shoot inducing medium. Callus initiation from untransformed leaf explants of the commercial tobacco cultivars TL33, J6, A4 and 20/19 was inhibited when levels of kanamycin of 100 mg/l and higher were included in the callus inducing medium (Table 3.1). In this table, no cultivar difference in response to kanamycin or asulam is shown. The concentration of 100 mg/l of kanamycin could therefore be used to assay the transgenic tobacco plants for the presence of the *nptII* gene in a callus induction assay.

Similarly, rooting of untransformed tobacco plantlets of all four South African tobacco cultivars (TL33, J6, A4, 20/19) as well as the cultivar Samsun, was inhibited when 100 mg/l of kanamycin was included in the rooting medium (Table 3.1). This level of kanamycin could therefore be used to assay the transgenic tobacco plants

Table 3.1. The effect of kanamycin and asulam on the growth and development of untransformed tobacco *in vitro*.

Assay system[a]	Concentration[b] (mg/l)				
	0	20	50	100	200
Kanamycin					
Adventitious shoot initiation from leaf discs	+ + +	+ + +	+ +	-	-
Callus induction from leaf discs	+ + +	+ + +	+	-	-
Rooting of plantlets	+ + +	+ + +	+	-	-
Bleaching of leaf discs	-	-	-	+ + +	+ + +
Asulam					
Adventitious shoot initiation from leaf discs	+ + +	+	-	-	-
Continued growth of callus	+ + +	+	-	-	-
Browning of leaf discs	+ + +	+	+ + +	+ + +	+ + +

[a] There was no cultivar difference in the *in vitro* response of tobacco to kanamycin. A cultivar difference in the response of tobacco to asulam was observed. J6 was slightly more resistant to asulam than A4, TL33 or 20/19.

[b] Inclusion of kanamycin or asulam in tisssue culture medium.

- = response absent, + = response slightly present, + + = response present, + + + = response strongly present.

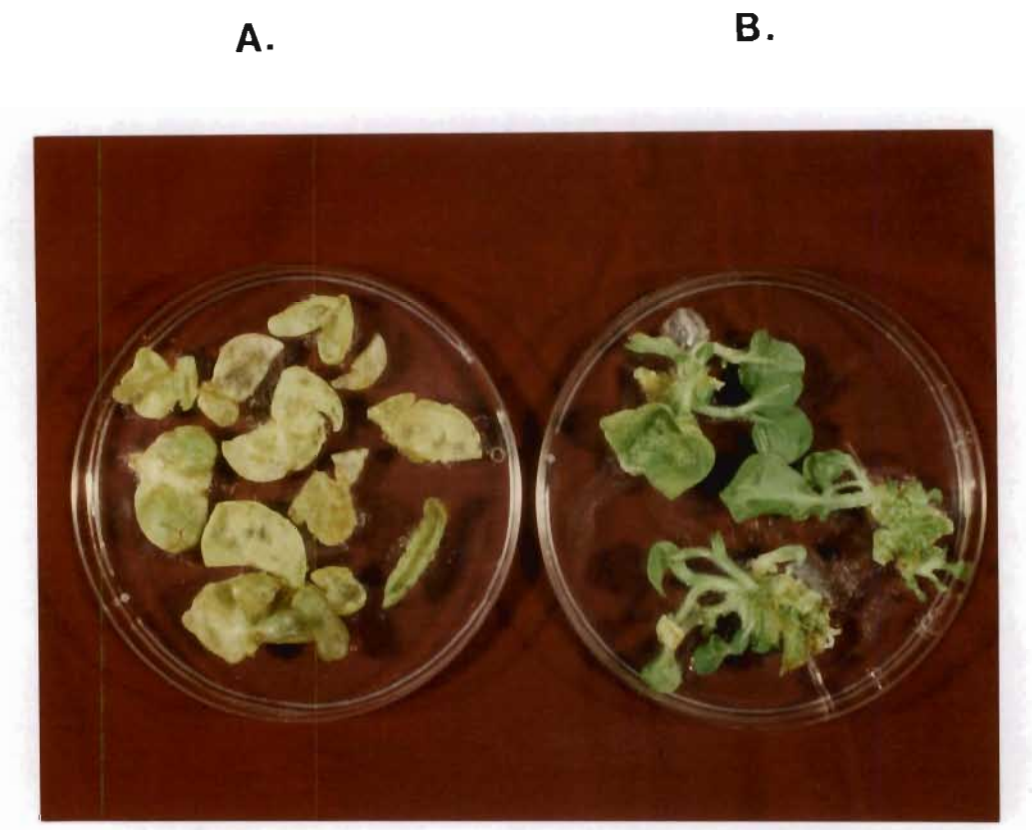


Fig. 3.1. The effect of kanamycin (100 mg/l) on untransformed tobacco leaves *in vitro*. (A) Untransformed tobacco leaf pieces on shoot inducing MS medium containing kanamycin. (B) Untransformed tobacco leaf pieces on shoot inducing MS medium without kanamycin.

for their ability to root in the root inducing MS medium containing kanamycin. Transgenic tobacco plants possessing the *nptII* gene were not able to root on this medium. Again, no cultivar differences are indicated in Table 3.1.

Leaf explants from untransformed tobacco cultivars 20/19, J6, TL33 and Samsum were exposed to shoot inducing medium containing between 0 mg/l and 200 mg/l of asulam (Table 3.1). The explants bleached initially and then turned brown. Adventitious shoot production was completely inhibited. A slight cultivar difference in the response to asulam was observed. The cultivar J6 was more tolerant to asulam at 50 mg/l than A4, 20/19, TL33 or Samsum. Asulam at a concentration of 150 mg/l was chosen to test transgenic tobacco plant material for expression of the *sul* I gene. Only transgenic plant material expressing the asulam resistance gene produced adventitious shoots on shoot inducing medium containing 150 mg/l of asulam.

Cefotaxime had little effect on tobacco adventitious shoot production and callus induction *in vitro* (Table 3.2). Also, cefotaxime controlled *Agrobacterium* at a minimum level of 250 mg/l. As a routine practice, however, 1000 mg/l of cefotaxime added to the tissue culture medium gave reliable control of this bacterium.

3.3.3 Isolation and culture of tobacco mesophyll protoplasts. Tobacco leaf mesophyll protoplasts were readily isolated from cv. White Burley leaves. Freshly isolated protoplasts suspended in MSP,9M medium are shown (Fig. 3.2). The protoplasts were stained with fluorescein diacetate and viewed under UV light to assess their viability following isolation. In a single field of view approximately 87% of the protoplasts were viable (Fig. 3.3). After several days, the protoplast cell walls regenerated and cell division occurred. Although no whole plantlets were regenerated from tobacco protoplasts, regeneration was carried out to the microcallus stage (Figure 3.4).

3.3.4 Direct DNA transformation of tobacco mesophyll protoplasts. The direct DNA transformation methods electroporation and polyethylene glycol treatment were successfully carried out. These techniques require careful optimization of

Table 3.2. The effect of cefotaxime on the growth and development of untransformed tobacco *in vitro*, and on the growth of *Agrobacterium tumefaciens*.

Assay system	Cefotaxime concentration[a] (mg/l)			
	0	250	500	1 000
Adventitious shoot initiation from leaf discs	+ + +	+ + +	+ + +	+ + +
Callus induction from leaf discs	+ + +	+ + +	+ + +	+ + +
Growth of *A.tumefaciens* on MS/LA medium	+ + +	+	-	-

[a] Inclusion of cefotaxime in tissue culture medium or bacterial growth medium

- = response absent, + = response slightly present, + + + = response strongly present

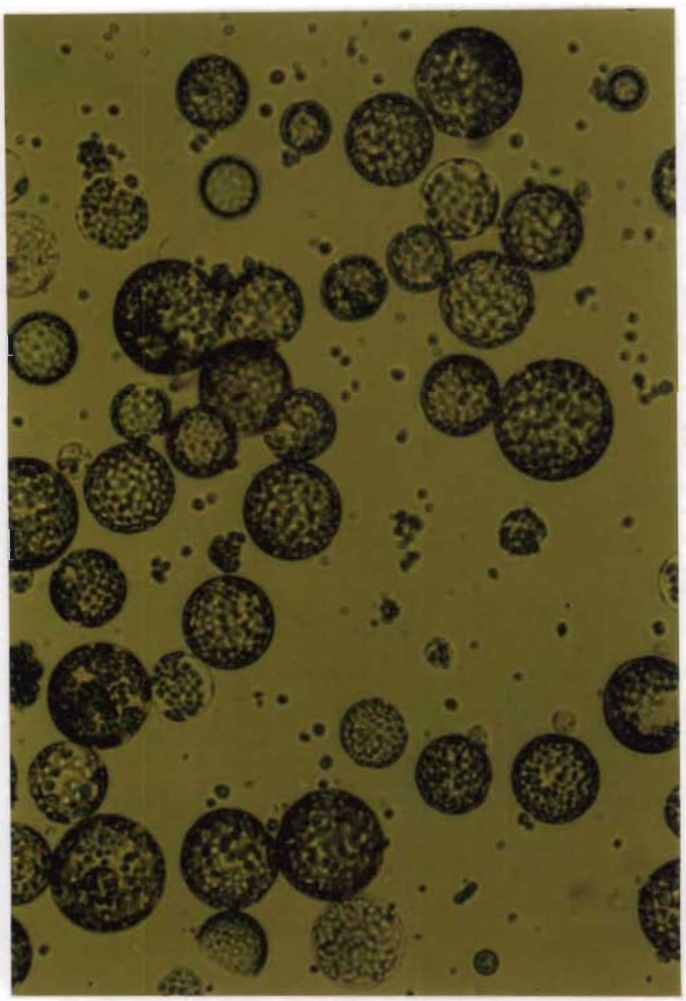


Fig. 3.2. Freshly isolated tobacco leaf mesophyll protoplasts (x 200).

Fig. 3.3. Fluorescein diacetate viability staining of tobacco mesophyll protoplasts. Viable protoplasts fluoresce, emitting various intensities of light, while the dead protoplasts do not emit any fluorescence (x 200).

Fig. 3.4. Microcalli regenerated from tobacco mesophyll protoplasts.

experimental conditions in order to achieve transformation.

3.3.4.1 Electroporation of tobacco mesophyll protoplasts. The electroporation conditions which favoured a 50% survival rate of tobacco protoplasts following electroporation were found to be a capacitance of 2770 μF, a voltage of 60 V (field strength 300 V/cm) and a pulse duration of 10 milliseconds (msecs). Table 3.4 shows the effect of various electroporation voltages on protoplast survival. At electroporation voltages of greater than 60 V most of the protoplasts were shattered (Fig. 3.5). Voltages higher than 60 V were therefore not suitable for the electroporation of tobacco mesophyll protoplasts.

3.3.4.2 Polyethylene glycol (PEG) treatment of tobacco mesophyll protoplasts. It was found that treatment of the tobacco protoplasts with 20% PEG gave a high survival rate (50%) of the protoplasts (Table 3.3). This concentration of PEG was used for the direct transformation of tobacco protoplasts with vector DNA.

3.3.4.3 Extraction of tobacco mesophyll protoplast DNA and DNA dot-blot analysis. DNA was isolated from 500 μl amounts of electroporated protoplasts, as well as from 1 ml amounts of PEG-treated protoplasts. Following the dot-blotting procedure and the probing of the blot with a DIG-labelled whole plasmid (pJIT119) probe, small areas of hybridization could be observed. A serial dilution of pJIT119 hybridized very strongly to this probe (Fig. 3.6). The DNA dot blot revealed that electroporation and PEG treatment with 20% PEG allowed the entry of small amounts of foreign DNA into the protoplasts.

3.3.4.4 Assay of transient GUS activity in tobacco mesophyll protoplasts. Protoplasts were assayed for transient GUS activity 24 hours and 48 hours after electroporation and PEG treatment using the histochemical assay (Jefferson, 1987). No GUS activity was observed in these cells.

3.3.5 Tobacco single cell culture inititation. The tobacco cultivar 20/19 was successfully established first as callus, and then as a callus culture, and then as a single cell culture. After several subcultures, the single cell culture consisted of a

Table 3.3. The effect of electroporation voltage and polyethylene glycol concentration on the survival of tobacco mesophyll protoplasts.

Treatment	Protoplast survival (%)[a]
Electroporation[b] (voltage)	
10	100
20	100
60	50
80	5
100	0
Polyethylene glycol (g/100 ml)	
0	100
5	100
10	80
20	50

[a] Determined by cell counting using a haemocytometer
[b] Electroporation at a capacitance of 2770μF and a pulse duration of 10 msecs

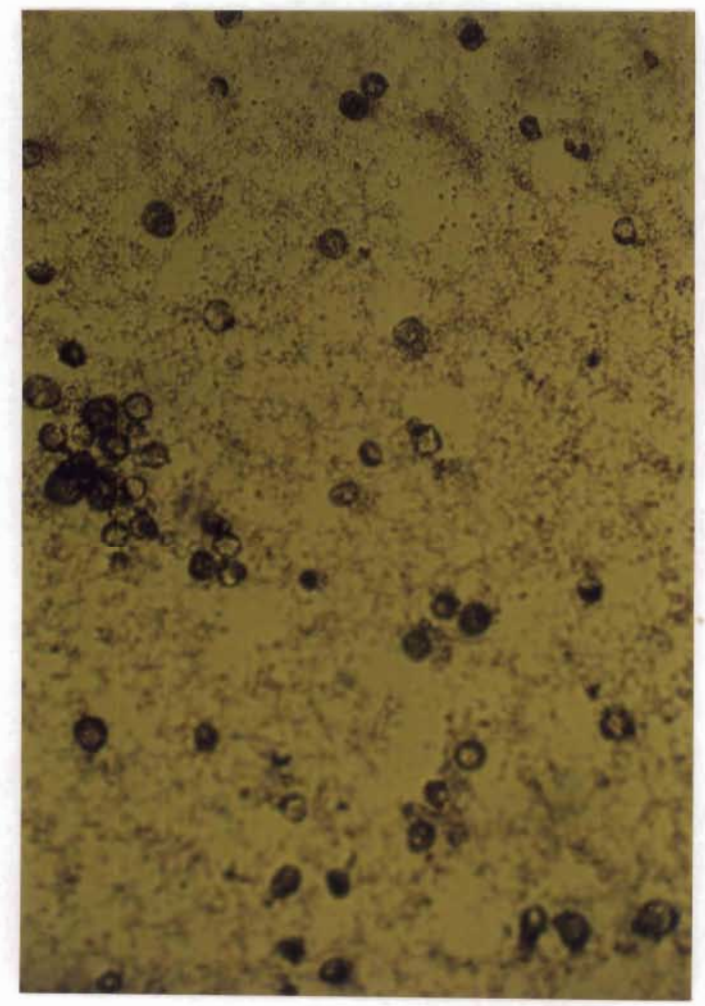
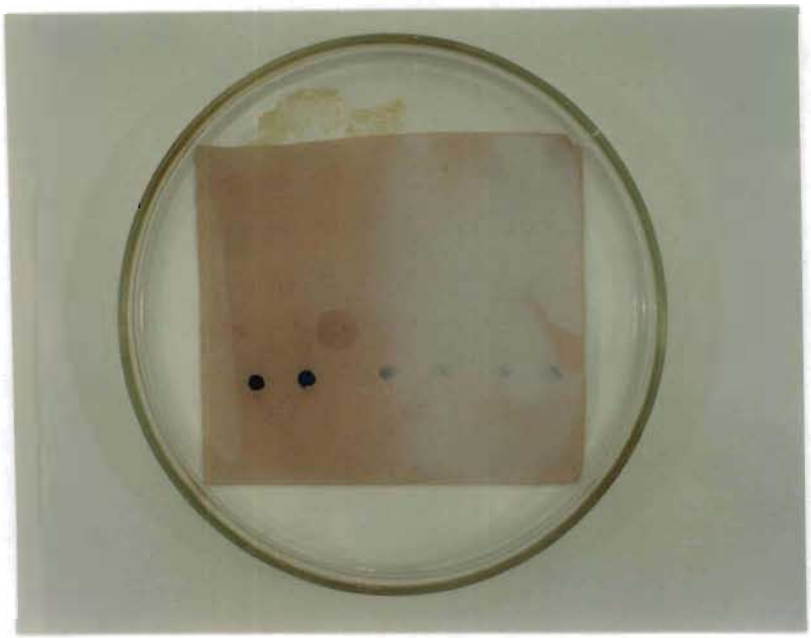
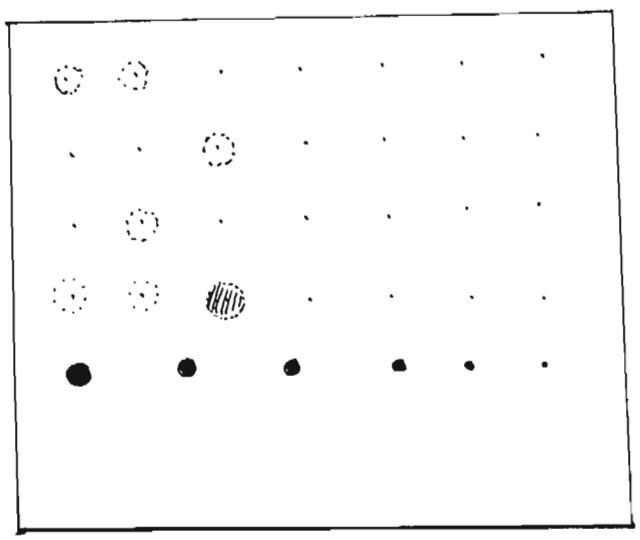


Fig. 3.5. Tobacco mesophyll protoplasts following treatment with a lethal electroporation voltage (80 V). More than 50% of the protoplasts were destroyed (x 200).



A



B

Fig. 3.6. Dot-blot analysis of total DNA isolated from electroporated and PEG treated tobacco mesophyll protoplast samples. (A) Dot-blot showing hybridization of the DIG-labelled pJIT119 probe to foreign DNA within the protoplast samples. Some of the pencil positioning dots are surrounded by a halo of small dots which are the result of the hybridization of the probe to the foreign DNA. The dark blue spots correspond to a serial dilution of pJIT119DNA. In the protoplast samples, the amount of visible hybridization is very sparse owing to the tiny amounts of DNA involved. (B) Diagram of the dot-blot.

fine suspension of cells. Subculturing the cells every seventh day produced cell cultures in a vigorous growth phase. Tobacco shoots were successfully regenerated from untransformed tobacco single cell cultures following the plating out of these cells by embedding them in shoot inducing MS medium (Figure 3.7).

3.3.6 *Agrobacterium*-mediated single cell culture transformation. After the co-cultivation of tobacco single cell cultures of tobacco with *Agrobacterium*, it was difficult to prevent bacterial overgrowth of the cell culture. When the cefotaxime concentration was increased from 1 g/l to 1.5 g/l, the tobacco cells in the culture died. Tobacco single cell transformation was not pursued further.

3.3.7 *Agrobacterium*-mediated tobacco leaf disc transformation. Numerous transgenic tobacco plantlets were obtained following the application of the *Agrobacterium*-mediated leaf disc transformation method to the South African tobacco cultivars J6, A4, 20/19 and TL33, and the cultivar Samsun. After four weeks on shoot inducing medium containing kanamycin, small shoots developed from the cut edges of the tobacco leaf pieces. These shoots were excised after eight weeks and grown independently on selection medium containing kanamycin. The shoots were finally rooted in rooting medium containing kanamycin (100 mg/). Plants which did not root at this stage (about 20% of the plantlets) were discarded. These transgenic plants were all produced with *Agrobacterium tumefaciens* transconjugant strain C58C1(pGV2260)(pJIT119). The main stages of tobacco leaf development during a leaf disc transformation experiment are shown in Fig. 3.8.

Repeated selection of plantlets on shoot inducing medium, as well as rooting of the plantlets on root inducing medium containing 100 mg/l kanamycin successfully identified "escape" tobacco plantlets. "Escape" plants are plants which initially express the *nptII* gene, but subsequently fail to express this gene. These plantlets bleach when grown on shoot inducing medium containing kanamycin. They also fail to root on root inducing medium containing kanamycin. Approximately 20% of adventitious shoots arising following the transformation and regeneration procedures of a leaf disc experiment were found to be "escape" plants and were discarded. Fig. 3.9 illustrates the difference between a fully transgenic tobacco plantlet able to

Fig. 3.7. Tobacco shoots (cv. 20/19) regenerated from an aliquot of single cell grown in liquid culture medium. The cells were plated out in shoot inducing MS medium containing 0.6 % agar and cultured in the light. Microcalli can also be seen in this photograph.

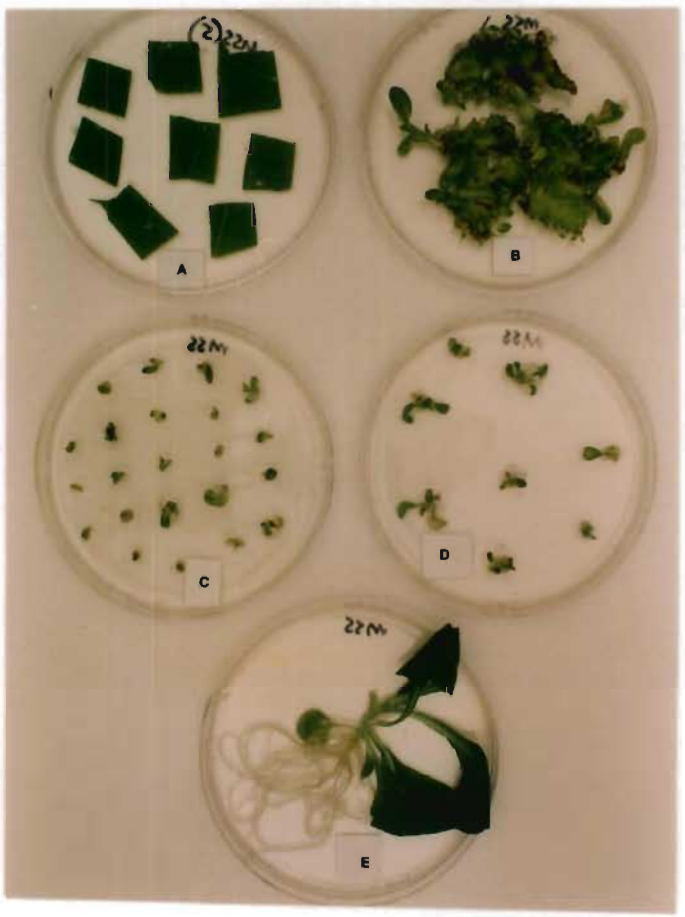


Fig. 3.8. The stages of the *Agrobacterium*-mediated leaf disc transformation method. (A) Tobacco leaf squares placed on shoot inducing medium prior to being inoculated with *Agrobacterium*. (B) Shoots developed along the cut edges of the leaf squares. Kanamycin included in the shoot inducing medium selected for transgenic shoots. (C) Small shoots were excised from the leaf pieces. (D) During several subcultures, the shoots increase in size. (E) Plantlets were rooted on rooting medium containing kanamycin. This plantlet can now be hardened off.

A. B.

Fig. 3.9. The difference between a transgenic tobacco plant and a plant that has "escaped" selection on kanamycin. (A) An "escape" tobacco plant showing the inability of this plant to root on kanamycin (100 mg/l). Several bleached leaves are also visible. (B) A transformed plantlet is able to produce roots on kanamycin (100 mg/l) and the leaves have a normal green colour.

root on medium containing kanamycin (100mg/l) and an "escape" plantlet which produced no roots.

3.3.7.1 Hardening off of *in vitro* grown transgenic tobacco plants. Ten hardened off plants, numbered 2 - 11, were selected for detailed evaluation. Details of these plants are listed in Table 3.4. Fig. 3.10 shows the hardened off transgenic tobacco plants. These transgenic tobacco plants were successfully grown to maturity. All plants flowered and set seed.

3.3.7.2 DNA dot-blot analysis of tobacco DNA. Genomic DNA was successfully extracted from transgenic tobacco leaves. From each 1 g of leaf material, between 70 - 150 μg of DNA was obtained. The appearance of the final pellet was gelatinous, presumably because of the high carbohydrate content. Treatment with methoxyethanol reduced the amount of the gel, but also reduced the DNA yield by approximately 30%. The plant DNA ran as a compact band at approximately 21 kb on a 0.8% seakem agarose gel, showing that there was no appreciable degradation.

A DNA dot-blot of genomic DNA isolated from individual transgenic tobacco plants probed with a radioactively labelled 2.7 kb pGV941 T-DNA probe revealed positive hybridization of the probe to all samples (Fig. 3.11). The radioactive probe did not hybridize to DNA from untransformed tobacco plants. The DNA dot-blot analysis confirmed the presence of a foreign T-DNA insert into the genomic DNA of each transgenic tobacco plant. The DNA sample from the control untransformed plant material (cv. TL33) did not hybridize to the T-DNA probe from pGV941.

Table 3.4. Transgenic tobacco plants selected for further study.

Transformed tobacco plant code no.	Cultivar
# 2	TL33
# 3	J6
# 4	TL33
# 5	J6
# 6	J6
# 7	Samsun
# 8	20/19
# 9	J6
# 10	J6
# 11	TL33

Fig. 3.10. Hardened off transgenic tobacco plants.

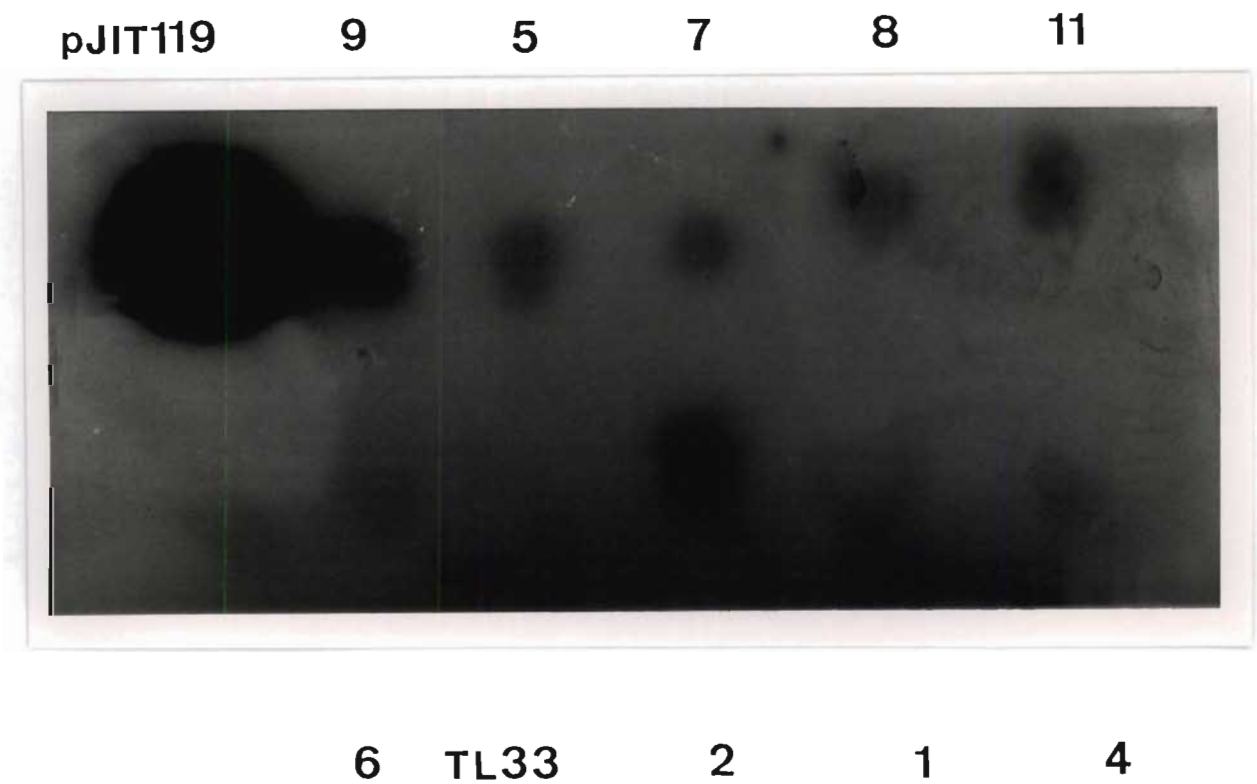


Fig. 3.11 DNA dot blot of transgenic tobacco. Genomic DNA isolated from transgenic plants 2, 3, 4, 5, 6, 7, 8, 9 and 11 was probed with a radioactively labelled T-DNA probe. Genomic DNA from an untransformed plant (cv. TL33) served as a negative control. DNA of pJIT119 served as a postive control. The T-DNA probe hybridized with the plasmid control DNA, and all the transgenic plant DNA samples. There is no hybridization of the probe to the untransformed TL33 DNA sample.

3.4 Discussion

Transgenic plants from cultivars TL33, 20/19 and J6 and the cultivar Samsun, expressing genes from the binary vector pJIT119 were successfully obtained following *Agrobacterium*-mediated leaf disc transformation. All South African tobacco cultivars performed equally well *in vitro* and in the leaf disc transformation experiments. No special optimization had to be performed in order to elicit a satisfactory regeneration or transformation response in any of the cultivars. Selected plants were coded 2 - 11 and were used in further studies to investigate foreign gene expression and inheritance.

Many crop plant species show genotype variation in their tissue culture and transformation response. The variation in tissue culture and transformation response can be as great between cultivars as that between species (Paterson and Everett, 1985; Power, 1987). Fortunately, cultivar differences were not obvious when the *in vitro* regeneration of shoots from tobacco leaf discs of South African tobacco cultivars 20/19, TL33 and J6 or Samsun was carried out. There were also no cultivar differences in the response of the tobacco cultivars 20/19, TL33, J6 and Samsun to the *Agrobacterium tumefaciens* strain C58C1(pGV2260)(pJIT119). The genetic engineering of South African tobacco cultivars 20/19, TL33 and J6 had not previously been carried out, and therefore this investigation established that the procedures reported in the literature for the transformation and regeneration of tobacco (Draper *et al.*, 1988; Horsch *et al.*, 1988), were suitable for the South African cultivars.

In carrying out *Agrobacterium*-mediated transformation of tobacco plants, care was taken to identify "escape" plantlets. Tobacco plantlets identified as having "escaped" from initial selection had either lost the *nptII* gene, or could not express the gene, and were of no further use as genetically engineered plants.

Once plantlets have been confirmed as transgenic, they were transferred to soil as soon as possible. This procedure eliminated the need for the continual subculturing of the plantlets *in vitro*, and allowed the plants to reach maturity and flower. The

production of fertile transgenic plants which set seed is an essential part of the process of producing transgenic plants. The presence of foreign genes in the progeny is final proof that the original transgenic plants were stably transformed. Transgenic seeds can be used to produce seedlings for greenhouse or field trials, and are a convenient way of storing transgenic material for future use. Also, the risk of somoclonal variation occurring is reduced if plants are maintained in tissue culture for as short a period as possible. Somoclonal variation is caused by mutations which occurs in plants after prolonged culture *in vitro* (Evans and Sharp, 1986; Evans *et al.*, 1987).

Tobacco mesophyll protoplasts were sucessfully obtained and cultured to the microcallus stage. Electroporation and PEG-mediated DNA transfer systems were applied to freshly isolated protoplasts, but no transient GUS activity could be observed in the protoplasts using the GUS histochemical method. The careful optimization of all parameters involved in the use of electroporation or PEG treatment for the transformation of plant protoplasts was essential. The expression of transient GUS activity was an aid to this optimization, as was the hybridization of DNA isolated from protoplasts with a DIG-labelled probe following direct DNA transfer. Following electroporation and PEG DNA transfer to tobacco leaf protoplasts, no GUS activity could be detected using the GUS histochemical assay (Jefferson *et al.*, 1987). The more sensitive fluorimetric GUS assay (Jefferson *et al.*, 1987) may have detected the low levels of GUS activity in the protoplasts. It is possible that a poor survival rate of the protoplasts over a 24-48 hour period following electroporation and PEG treatments could have contributed to the failure of the GUS gene to express. The probing of protoplast DNA with a DIG-labelled pJIT119 whole plasmid probe showed that the pJIT58/1 DNA entered the protoplasts following the optimized electroporation and PEG treatments. The binary vector pJIT119 has many regions of homology with pJIT58/1, allowing it to be used as a useful probe for pJIT58/1 DNA.

The isolation and transformation of tobacco mesophyll protoplasts is a demanding task. Where alternate methods of plant transformation exist, these should rather be pursued. Plant protoplasts, nevertheless, have a major role to play in plant genetic

engineering for plant improvement. In genetic engineering, many individual transformation events can be generated through the electroporation or PEG treatment of protoplasts in the presence of DNA. Also, to aid the development of new vector constructs, the assay of transformed protoplasts for transient expression of foreign genes within 48 hours can give an indication of whether or not a new vector construct is expressed in a plant system. In addition, other important uses of plant protoplasts include protoplast fusion and the microinjection of DNA into protoplasts.

Tobacco single cell cultures were readily established from callus derived from the South African tobacco cultivar 20/19. The rapid growth rate of these cultures can provide plant material for numerous transformation experiments. From the co-cultivation of tobacco single cells with a vector-containing *Agrobacterium* strain, many thousands of transgenic plantlets can be obtained. The tobacco cells should not be maintained in culture for long periods of time, as the nature of the cells will change due to somaclonal variation.

A number of transgenic tobacco plants of South African cultivars were produced via the *Agrobacterium*-mediated leaf disc transformation method for further study and evaluation. While the more complex methods of transformation have important uses, the *Agrobacterium*-mediated leaf disc transformation method proved to be the method of choice for the routine production of trangenic plants from South African tobacco cultivars.

Chapter 4

An analysis of foreign gene expression in transgenic tobacco plants

Chapter 4

An analysis of foreign gene expression in transgenic tobacco plants

4.0 Summary

Initial observations on the group of transgenic tobacco plants transformed with the binary vector pJIT119, revealed that each plant expressed the *nptII* gene from pJIT119, but seldom expressed the *sul* I and *uidA* genes from this vector as well. Foreign genes not under selection were, therefore, inactivated at a high frequency in these transgenic tobacco plants. This high level of inactivation made a detailed examination of foreign gene expression in the plants necessary. A number of simple assays were carried out to establish the foreign genes which were expressed in each individual transgenic plant. In addition to these assays, computerized image analysis was carried out. Image analysis was used to quantify the growth of transgenic tobacco callus in response to *in vitro* culture on medium containing the herbicide asulam. Several different quantitative assays for neomycin phosphotransferase (NPTII) activity in extracts of transgenic tobacco plants were also carried out.

4.1 Introduction

Most plant genetic engineering experiments aim to create plants with a measurable change in the plant phenotype, mainly through the introduction and expression of novel foreign genes (Dunsmuir *et al.*, 1988). However, as there is a high level of variation in the copy number and expression of introduced genes, the transgenic plants produced in a transformation experiment can differ with regard to foreign gene presence and expression (Dunsmuir *et al.*, 1988). For agricultural improvement, transgenic plants expressing the highest levels of a novel foreign gene must identified for further evaluation.

The Ti plasmid based vectors designed for plant genetic engineering possess a number of features which aid the study of foreign gene expression in transgenic plants. The selectable marker genes contained by these vectors assist in the selection of transgenic plant material from a background of non-transgenic material. Selectable resistance is achieved through the introduction of genes for antibiotic or herbicide resistance into the plant material. However, selectable marker genes can also be quantitatively assayed to provide an analysis of levels of foreign gene expression in individual transgenic plants. The selectable marker gene most commonly used in plant genetic engineering is *nptII*, encoding the enzyme neomycin phosphotransferase (NPTII). The enzyme NPTII confers kanamycin resistance when introduced into plants. The activity of this enzyme can readily be assayed in plant material. The screenable marker gene, *uidA*, is also a useful gene for identifying transgenic plant material. The activity of the gene product, ß-glucuronidase (GUS), can also be assayed quantitatively (Reynaerts *et al.*, 1988).

However, regardless of the ease of assay of the products of traditionally used selectable and screenable marker genes, the translational products of many foreign genes cannot be assayed by biochemical means. The assay of those foreign genes which have agricultural value in the whole plant (eg. foreign genes for insect resistance, disease resistance and cold tolerance) present a challenge. The presence of the foreign gene products can be detected through the use of methods like Western blotting, but the effectiveness of the gene products in conveying the

desired transgenic phenotype must be assayed by less convenient methods. In these situations, living tissue or whole transgenic plants must be challenged with an insect pest or infectious agent either *in vitro*, in the greenhouse or in the field. Similarly, changes to other characteristics relating to the end use of a transgenic crop (eg. ripening characteristics) have to be assayed in mature plants.

From the plant gene vector, pJIT119, a transgenic plant receives T-DNA containing two selectable marker genes, *nptII* and *sul* I, and a screenable marker gene (*uidA*). Both the *nptII* and *uidA* genes produce a gene product which is easily quantifiable, neomycin phosphotransferase and ß-glucuronidase respectively. There is as yet no convenient assay available for the product of the *sul* I gene, dihydropteroate synthase. Quantification of the expression of this foreign gene can only be determined by exposing either *in vitro* grown plants or mature plants to the herbicide asulam.

During investigations of foreign gene expression in transgenic tobacco plants, the failure of the *nptII* gene to be expressed was often encountered. The *nptII* gene is commonly studied with reference to "escape" plants because this gene is heavily relied on to distinguish transgenic from non-transgenic plants (Draper *et al.*,1988; McHughen and Jordan, 1988; Jordan and McHughen, 1989). Plants which have been through a genetic engineering process, and which initially appear transgenic, but which are later found to not express the *nptII* gene, are known as "escapes". Usually, a plant which has "escaped" selection contains the *nptII* gene, but there is no expression of this gene. The "escape" plants studied by Horsch *et al.* (1988) suffered a loss of foreign gene expression, rather than a loss of the foreign DNA sequences. However, "escape" plants may also sometimes no longer contain foreign DNA. In transgenic plants produced by Blake *et al.* (1991), the *uidA* gene was physically lost from 28% of their transgenic plants during the transformation process (McHughen and Jordan, 1989). Although "escapes" in the *nptII* gene are most often reported on, "escapes" in other foreign genes can occur just as easily. Blake *et al.* (1991) report that their efforts to transform plants with a *nptII* gene linked to the *uidA* gene resulted in "escapes" in the *uidA* gene as well as in the *nptII* gene. Deroles and Gardner (1988) also reported a 16% loss of expression of either of two chimeric

genes in transgenic plants when two genes were present in the original T-DNA.

DNA methylation, as well as the "position effect" (the location of the foreign gene within the plant genome), can cause the foreign gene inactivation which is manifested as "escape" plants. Deletions which occur during the original T-DNA integration event could cause gene losses from the T-DNA. The loss of expression of certain transgenes from a group of transgenic tobacco plants was considered in the light of these phenomena.

Integration of T-DNA into the plant genome is considered to be an efficient process, but despite this, deletions and rearrangements often occur, particularly near the left- and right-hand border sequences of the T-DNA molecule. DNA rearrangements can occur during the initial integration event or during subsequent mitotic divisions (Scott *et al*., 1988; Blake *et al*., 1991).

The methylation of cytoxine residues in DNA is involved in the regulation of gene expression in plants and bacteria (Palmgren *et al*., 1993). In plants, up to 30% of all cytosines are methylated and the level of DNA methylation in plants is modulated during development and differentiation. DNA methylation may cause foreign gene inactivation through *de novo* methylation of previously unmethylated sequences (Weber *et al*., 1990; Palmgren *et al*., 1993). The methylation of foreign genes in plants will block foreign gene transcription, resulting in the absence of the expected gene product. The application of 5-azacytidine (azaC), either to *Agrobacterium* in culture, or to transgenic plant tissue in culture, can reactivate a methylated introduced gene (Bochardt *et al*., 1992). The application of 5-azacytidine to plant cells is an efficient way to recover transgene expression, but since other endogenous plant genes my also be affected in an unpredictable way, it is not generally practical (Palmgren *et al*., 1993).

The position effect, or the effect of different sites of foreign gene insertion into the plant genome, can affect the absolute level of foreign gene insertion. Different regions of the plant genome are expressed at different levels and at different stages in the plant life cycle (Deroles and Gardner, 1988). Foreign genes which are inserted

in an inactive region of the plant genome will not be expressed.

Several sensitive assays are available to assess the activity of the neomycin phosphotransferase (NPTII) enzyme in transgenic plants. In plant systems, the enzyme activity as a result of the integration of *nptII* into the plant genome is low, and this presents a challenge for the sensivity of NPTII assays (Cabanes-Bastos *et al.*, 1989). The NPTII enzyme is more easily assayed in prokaryote systems where the levels of enzyme are usually high. Plant cells also differ from bacterial cells in that they contain high levels of endogenous ATPase activity which competes for the radioactive ATP used in the NPTII assays, causing a reduction in the sensitivity of the assays (Cabanes-Bastos *et al.*, 1989). For this reason, there have been a number of extremely sensitive assay systems developed. Reiss *et al.* (1984) published a method requiring non-denaturing polyacrylamide gel electrophoresis (ndPAGE) and radioactive ATP to detect neomycin phosphotransferase activity. Cabanes-Bastos *et al.* (1989) improved on this method, devising a convenient method to assay for the activity of neomycin phosphotransferase in crude plant cell extracts using thin layer chromatography. Both these assay methods require the use of radioactive ATP and specialized facilities for working with radioactive compounds. However, the method of Cabanes-Bastos *et al.* (1989) requires less radioactive ATP and has fewer steps, and therefore is the more convenient method. In this present study, extracts from transgenic tobacco were assayed using both these methods.

Recently, another neomycin phosphotransferase assay method was published which employed paper chromatography (Roy and Sahasradbudhe, 1990). The authors claimed that this method required less radioactive ATP than other published methods, which no doubt is advantageous in terms of cost and safety. There is also an enzyme linked-immunosorbent assay (ELISA) method for the rapid detection and quantification of neomycin phosphotranferase II in transgenic plants (Nagel *et al.*, 1992). These two methods were not investigated in this study.

In the absence of a quantitative assay for the *sul* I translation product, dihydropteroate synthase, computerized image analysis was utilized to measure

differences in the growth response of transgenic tobacco callus cultured on tissue culture medium containing asulam. The image analyzer was able to measure several parameters related to growth *in vitro* over a period of time. The diameter and perimeter of a sample could accurately be measured, and related to an increase in size of the sample.

The growth of plant tissues *in vitro* involves change on a very small scale. To quantify growth *in vitro*, researchers usually rely on destructive methods of measurement, chiefly the determination of dry or fresh weight. Dry weight measurement obviously renders each tissue sample non-viable. Wet weight measurement is more satisfactory, as the sample is replaced in the *in vitro* environment after weighing, but careful aseptic weighing methods are required. The transfer of the tissue during weighing may also disturb the tissue, influencing the results of an experiment (Smith *et al.*, 1989). For the indirect measurement of plant growth *in vitro*, other methods, including image analysis, have been sought. Image analysis has been found to be an adequate substitute for fresh weight measurement (Mottley and Keen, 1987).

The image analysis video camera and computer system is used to gather (film) and compute data on the area (mm^2) and width (mm) of a sample growing *in vitro* over a period of time. The measurements can be taken within the culture vessel. The data provides an estimate of growth without the need to remove the explant from the culture environment. The method is non-destructive and as the sample is measured within the culture vessel, allows the repeated scanning of the same plant explant without disturbance or contamination Subtle time course changes in plant growth rate can be recorded by this method (Mottley and Keen, 1987; Smith *et al.*, 1989; Coles *et al.*, 1991).

The preparation of transgenic tobacco plants from South African commercial tobacco cultivars necessitated the use of selectable and screenable marker genes, mainly the *nptII* gene, the *sul* I gene and the *uidA* gene. Simple, convenient assay methods, including the callus induction assay, growth of transformed plantlets on medium containing the herbicide asulam, and the GUS histochemical assay

provided valuable information on the expression of these marker genes in individual transgenic tobacco plants.

4.2 Materials and Methods

4.2.1 Identification of transgenic tobacco plants. To confirm that tobacco plants were transgenic, the continual selection of plantlets on shoot inducing and root inducing tissue culture medium containing kanamycin (100 mg/l) was carried out. This enabled transformed plantlets which expressed the *nptII* gene to be distinguished from those "escape" plants which did not. Plants which were green and produced shoots or roots on MS medium containing kanamycin were considered to be transgenic. Plants which were identified as non-transgenic "escapes" were discarded. Plants which failed to produce shoots or roots on MS medium containing kanamycin, or which were bleached, were considered to be non-transgenic. Only the plantlets expressing the *nptII* gene were retained for further study.

To further confirm that the transgenic tobacco plants numbered 2-11 possessed an active *nptII* gene, the callus induction assay was performed. To carry out the callus induction assay, callus induction MS medium (Murashige and Skoog, 1962) was prepared containing 100 mg/l kanamycin. Leaves of individual transgenic tobacco plants cultured *in vitro* were cut into 1 cm^2 squares and cultured on the callus induction medium, at 26 °C, in the dark. Only leaf material expressing an *nptII* gene developed callus on this medium under these conditions. This assay was used to confirm that the plants 2-11 expressed a functional *nptII* gene, and that all "escape" plants had been discarded.

4.2.2 Analysis of the transgenic phenotype of transformed tobacco plants. The tobacco plants 2 - 11 were confirmed as transgenic because they contained an active *nptII* gene. These plants were analyzed further to determine which other two foreign genes, *sul* I and *uidA*, derived from pJIT119, they expressed. To determine the expression of a functional *sul* I gene in these plants, leaf explants from plants 2 - 11 were cultured on tissue culture medium containing asulam. To perform this, 1 cm^2 pieces of transformed leaf material from each transgenic tobacco plant were placed on shoot inducing MS medium (Murashige and Skoog, 1962) containing 200 mg/l of the herbicide asulam (Maybaker). Incubation was in the light

(37.5 $\mu E.m^{-2}.s^{-1}$) at 26 °C. The presence or absence of shoots on this medium was recorded after six weeks of culture. The results were recorded photographically.

Callus derived from leaves of transgenic tobacco plants 2 - 11 was also cultured on callus inducing MS medium (Murashige and Skoog, 1962) containing asulam to test for its ability to proliferate in the presence of asulam. The callus from each transgenic plant, as well as from non-transgenic plants was cultured for six weeks on callus inducing MS medium containing a series of asulam concentrations (0 mg/l, 50 mg/l, 100 mg/l, 200 mg/l and 400 mg/l). The colour and size of the callus pieces was observed after six weeks and the results recorded photographically.

Samples from each of the plants numbered 2-11 were tested for *uidA* gene activity using the GUS histochemical assay (Jefferson, 1987). One centimeter lengths of stem and 1 cm^2 pieces of leaf from *in vitro* cultured tobacco plants numbered 2 - 11 were assayed for GUS activity. The results were recorded photographically.

4.2.3. Quantitative analysis of inter-transformant variation of foreign gene expression. In order to quantify foreign gene expression (*nptII*, *sul* I) in transgenic tobacco plants, image analysis and assays for neomycin phosphotransferase (NPTII) were carried out. Prior to this analysis, it had been noted that not all ten transgenic tobacco plants (plants 2 - 11) obtained from an *Agrobacterium*-mediated leaf disc experiment were fully resistant to asulam. Methods were sought to quantify the difference in asulam resistance. Computerized image analysis was used to quantify the *in vitro* response of selected individual transgenic plants to asulam. Transgenic plants 2, 4, 5, and 9, as well as untransformed cultivars were assayed. Also, two radioactive assays for neomycin phosphotransferase activity were carried out to assess their value in quantitatively determining foreign gene (*nptII*) expression in individual transgenic plants. Western blotting was also carried out to detect the NPTII problem in plant and bacterial extracts.

4.2.3.1 Computerized image analysis. To prepare plant material for image analysis, transgenic leaf pieces were cultured on callus inducing MS medium in the

dark at 26°C until callus was produced. Callus from transformed tobacco plants 2, 4, 5 and 9 was prepared in this way. Callus from untransformed tobacco cultivars TL33 and J6 was also prepared. Kanamycin (100 mg/l) was added to this medium to keep transgenic leaf and callus material under continuous antibiotic selection, but was omitted from the culture medium of non-transgenic material. The growth response of transformed and untransformed tobacco callus to the herbicide asulam was assayed by culturing pieces of callus on callus inducing medium containing a range of asulam concentrations *in vitro*. The asulam concentrations were 0 mg/l, 50 mg/l, 100 mg/l, 200 mg/l and 400 mg/l. Four pieces of callus of approximately equal size (visual estimation) from each transgenic plant were placed on each asulam concentration, in duplicate. The callus pieces were cultured on the asulam containing medium for four weeks in the dark at 26°C.

A Cambridge Quantimet 520 image analysis system, linked to an IBM compatible personal computer was used. For image capture, a Cambridge Instrument video unit fitted with a Tamron SP macro camera lens, supported by a Haiser RSI photographic stand with built-in illumination was used (Fig. 4.1). Each piece of tobacco callus was scanned through the base of the Petri dishes, minimizing the disturbance of the callus. A video image of each piece of callus was transmitted onto a computer screen. The computer then superimposed a digitised overlay over the image onto the computer screen (Fig. 4.2). When the overlay covered features which were not important in the computations, this information could be edited out with the built-in image editing function. The aim of the technique was to produce an overlay pattern which corresponded exactly to the two dimensional shape of the plant material under analysis.

The degree of asulam resistance of the pieces of callus was considered to be a function of the change in size of the callus pieces over a one month culture period. If the transgenic callus was resistant to asulam, it stayed the same size or increased in size. If the callus samples were not resistant to asulam, the samples might stay the same size or decrease in size.

After the callus pieces were placed in tissue culture to initiate the study, they

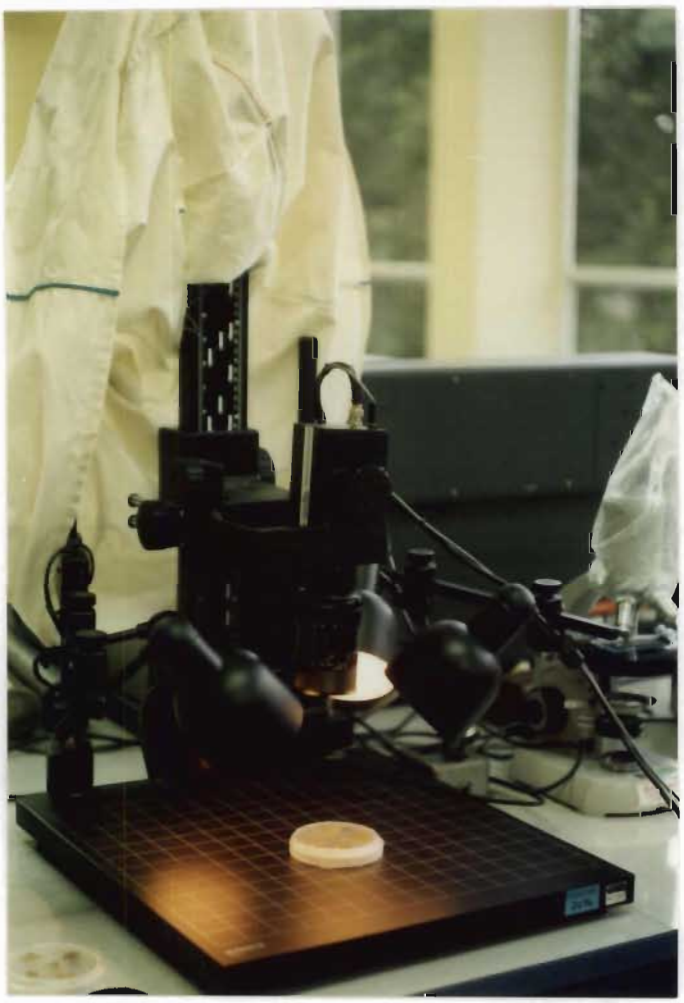


Fig. 4.1. The video camera unit of the Cambridge Quantimet 520 computerized image analysis system. Here the video unit is shown filming a sample of transgenic callus which is still contained inside the Petri dish.

Fig. 4.2. Computer and computer screen of the Cambridge Quantimet 520 image analysis system. The image of a plant sample was recorded using the image analysis video unit and displayed on the computer screen. The computer superimposes an overlay (red) onto the image of the sample displayed on the computer screen. From this overlay, the image analyzer is able to compute measurements of area (mm^2) and perimeter (mm) of the sample.

underwent an initial measurement (t_0) using the image analysis system. After a one month culture period, a second measurement was taken (t_1). The growth of each piece of callus was determined from the difference between these two measurements. The image analyzer measured the dimensions of the overlay, and computed both area (mm^2) and perimeter (mm) of the overlay. It must be noted that this area is not the actual surface area of the callus sample, but the area of the overlay.

The results for area (mm^2) were used to calculate the response of each sample to asulam and plot changes in response graphically. The mean size of callus explants ($n = 8$) from each treatment was determined at t_0 and at t_1. Then the value of $t_1 - t_0$ was calculated and plotted graphically. The Statpak regression and correlation programme "Correl" was used to test pairs of data for correlation and co-variance. The standard error for each set of data was determined.

4.2.3.2 Neomycin phosphotransferase assay. To detect active neomycin phosphotransferase II (NPTII) in extracts of transgenic tobacco tissue and bacteria, the radioactive methods of Reiss *et al.*, (1984) and Cabanes-Bastos *et al.*, (1987) were carried out. Western blotting was also carried out to detect the presence of the NPTII protein (Draper *et al.*,1988). The three methods were performed, evaluated and compared.

The method of Reiss *et al.* (1984) consisted of the preparation of a crude tissue extract and the fractionation of this extract on non-denaturing polyacrylamide gel electrophesis (ndPAGE) to separate NPTII from other proteins. The NPTII was detected *in situ* through its ability to phosphorylate kanamycin with [α-^{32}P] labelled adenosine triphosphate (ATP) (triethylammonium salt) in an agarose overlay. The presence of radioactive kanamycin phosphate was visualized by autoradiography. The reported range of detection was 5 - 10 μg of protein (Reiss *et al.*, 1984).

Cabanes-Bastos *et al.*(1989) developed a simpler and more rapid assay, based on the chromatographic separation of radioactive kanamycin-phosphate from the rest of the components of the reaction mixture on PEI-cellulose plates. The reaction

mixture consisted of a plant extract with [α-^{32}P] labelled ATP. Radiolabelled kanamycin phosphate was visualised by autoradiography. The reported range of detection is 0.5 pg - 500 ng of protein (Cabanes-Bastos *et al.*, 1989). PEI-cellulose (Polygram Cel 300 PEI) thin layer chromatography plates were purchased from Labotec, Industria, South Africa. The [α-^{32}P] labelled ATP was obtained from Amersham. An extract of untransformed tobacco (cv. J6) was used as a negative control. As a positive control, an active NPTII protein was extracted from *E. coli*(pGS9).

Protein concentrations in bacterial and plant extracts were determined using the Bio-rad protein assay method which is based on the method of Bradford (1976).

4.2.3.3 Western blotting. Western blotting was carried out to determine the amount of NPTII in a sample (Draper *et al.*, 1988; Peleman and De Clerq, 1989). Extracts of transgenic tobacco plants transformed with an *nptII* gene were separated by SDS PAGE gel electrophoresis. The separated proteins were transferred on to a nitrocellulose membrane through an electrical transfer system. The membrane was probed with an alkaline phosphatase conjugated anti-rabbit antiserum at room temperature for two hours. The amount of plant or bacterial protein which must be assayed in order to detect a specific protein is at least 50 ug (Draper *et al.*, 1988).

4.3 Results

4.3.1 Identification of transgenic tobacco plants. A small number of transgenic tobacco plants, numbered 2-11, rooted and maintained their green colour under *in vitro* kanamycin selection. These transgenic tobacco plants were considered to be stably transformed and were subjected to further detailed studies. The plants which "escaped" selection and were bleached or which failed to root on kanamycin were discarded.

Leaf samples from transgenic tobacco plants 2 - 11 cultured on callus inducing MS medium containing 100 mg/l kanamycin produced large amounts of pale-coloured callus. This callus could be propagated indefinitely on callus inducing MS medium containing kanamycin. Untransformed leaf material on this medium did not produce callus and the leaf samples eventually became bleached and moribund. The callus induction assay using kanamycin therefore confirmed that transgenic plants 2 - 11 contained an actively expressed *nptII* gene, and were therefore stably transformed.

4.3.2 Analysis of the transgenic phenotype of transformed tobacco plants. The response of leaf samples taken from transgenic plants 2 - 11 and cultured in the light on shoot inducing MS medium containing 200 mg/l asulam showed that each plant had a different response to asulam. Leaf samples from transgenic plants 4, 6, 7, 8, 10 and 11 turned brown in colour and failed to produce shoots when cultured on asulam (200 mg/l) (Fig. 4.3). These plants were therefore not asulam resistant *in vitro*, although they are transformed. Leaf samples from transgenic tobacco plants 2, 5 and 9 produced green adventitious shoots when cultured on asulam (200 mg/l). These plants were asulam resistant *in vitro* (Fig.4.3). The untransformed leaf samples (cv. TL33) rapidly died from their exposure to asulam (200 mg/l) (Fig. 4.3). As plants 2 - 11 were all transgenic, expressing the *nptII* gene, it was clear that many of the transgenic tobacco plants which expressed the *nptII* gene did not express the *sul* I gene.

Callus from individual transgenic plants (plants 2, 4, 5, 7 and 9), as well as callus from non-transgenic plants of cultivars J6, TL33 and Samsun, was cultured on callus

Fig. 4.3. *In vitro* leaf assay for asulam resistance. Leaf samples from individual transgenic tobacco plants were cultured on shoot inducing MS medium containing the herbicide asulam (200 mg/l). Transgenic plants 2, 5 and 9 produced normal green adventitious shoots when cultured on MS medium containing asulam. Leaf pieces from transgenic plants 3, 4, 6, 7, 8, 10 and 11 browned in response to asulam and failed to produce adventitious shoots. Leaf explants from an untransformed tobacco cultivar (cv. TL33) used as a control also failed to produce shoots on MS medium containing asulam.

inducing MS medium containing 0 mg/l, 50 mg/l, 100 mg/l, 200 mg/l and 400 mg/l asulam for four weeks. Callus from transgenic plants 2, 5 and 9 proliferated at all concentrations of asulam, and in some cases even underwent shoot organogenesis (Fig. 4.4). Callus from transgenic plants 4 and 7 failed to proliferate and eventually browned and decreased in size on all concentrations of asulam (Fig. 4.4). Untransformed callus from cultivars J6, TL33 and Samsun also failed to proliferate, turned brown and decreased in size on medium containing different concentrations of asulam. The cultivar J6 was slightly more tolerant of asulam than TL33 and Samsun (Fig 4.4). Tobacco plants 2, 4, 5, 7 and 9 were all transgenic, expressing the *nptII* gene.

Stem and leaf samples were taken from transgenic tobacco plants 2 - 11 and assayed for ß-glucuronidase (GUS) expression using the histochemical assay. The results of this assay showed that the transgenic plants were not all able to express the *uidA* gene (Fig. 4.5). Only plants 5, 8, 9 and 10 showed expression of the GUS gene. Transgenic plants 3, 4, 6, 7 and 11 did not express the *uidA* gene, although all expressed the *nptII* gene and were therefore transformed.

The survey of the expression of three foreign genes (*nptII*, *sul* I and *uidA*) from the binary vector pJIT119 in ten transgenic tobacco plants is summarized in Table 4.1. It can be seen that out of ten transgenic tobacco plants, only three expressed the *nptII*, *sul* I, and *uidA* genes from pJIT119, one plant expressed the *sul* I, and *nptII* gene, and six of the plants expressed the *nptII* gene, but expressed neither *sul* I nor *uidA*. This also means that six of the ten plants would be of no further use for commercial development since they did not contain the main gene of interest for crop improvement, the *sul* I gene. They were, however, still of interest for research purposes.

4.3.3 Quantitative analysis of inter-transformant variation in foreign gene expression.

4.3.3.1 Computerized image analysis. Image analysis was successfully applied to the study of the response of transgenic tobacco callus grown on tissue culture

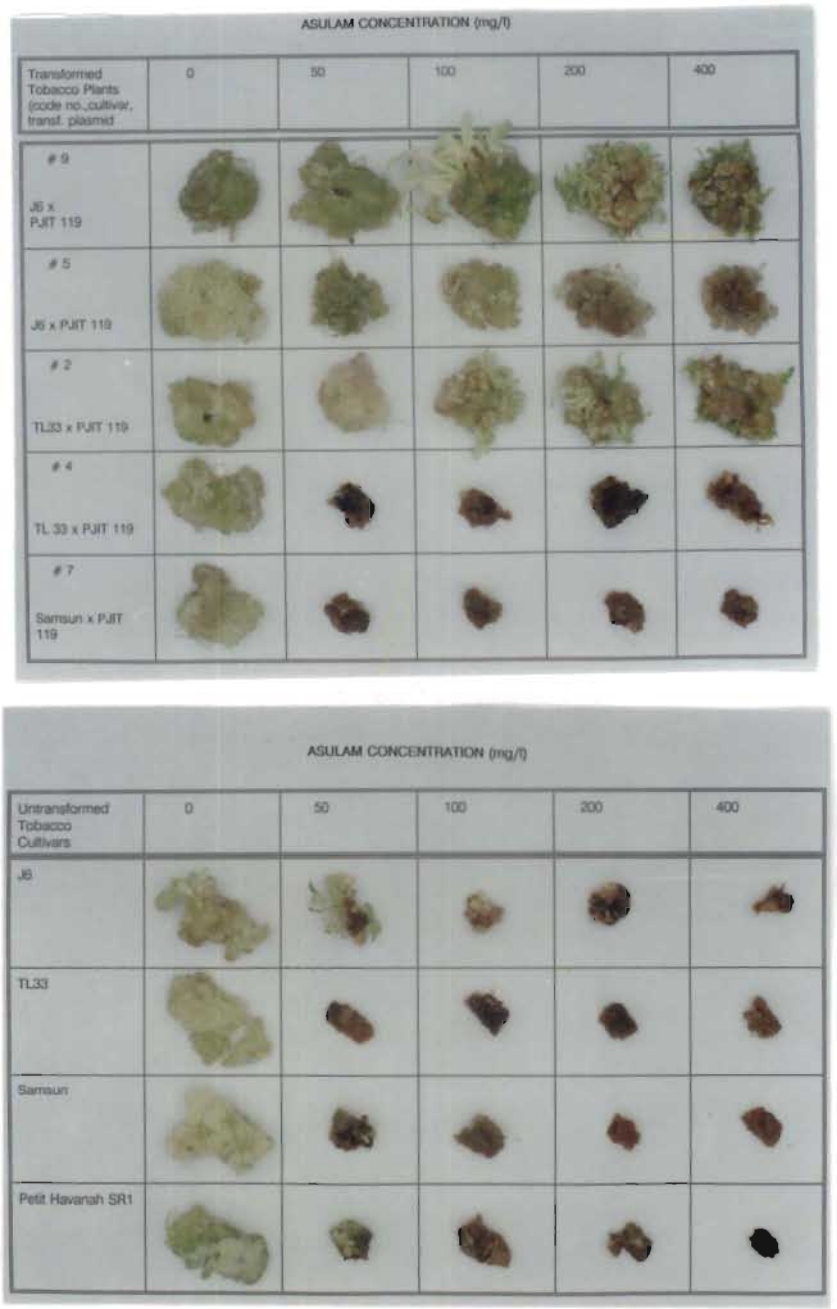


Fig. 4.4. Callus assay for asulam resistance. Pieces of callus from transgenic tobacco plants 2, 4, 5, 7 and 9 were assayed on callus inducing medium containing asulam 0 mg/l, 50 mg/l, 100 mg/l, 200 mg/l or 400 mg/l. Callus from untransformed tobacco (cultivars J6, TL33 and Samsun) was also assayed.

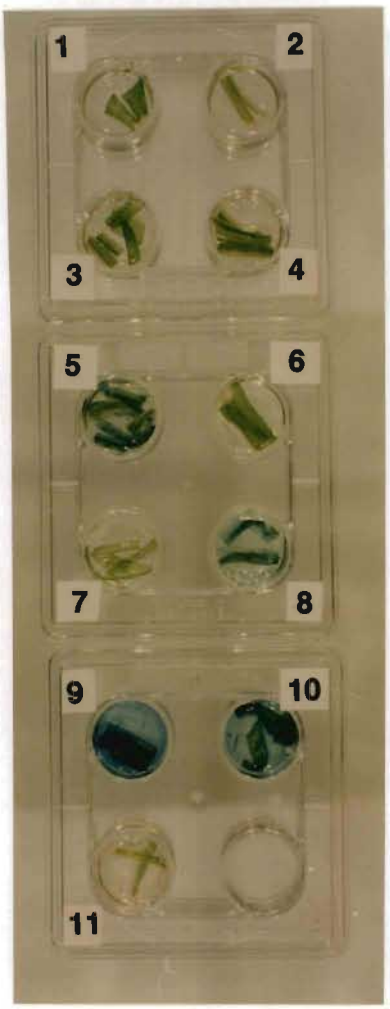


Fig. 4.5. Leaf and stem assay for ß-glucuronidase (GUS) activity. Stem and leaf explants from transgenic tobacco plants 2 - 11 were assayed. The numbers on the wells refer to individual transgenic plants. Well 1 contains an untransformed tobacco sample (cv. TL33, GUS negative). In transgenic plants 5, 8, 9 and 10 GUS activity is strongly evident. In transgenic plants 2, 3, 4, 6, 7 and 11 there is no GUS activity. GUS activity resulting from the expression of the *uid*A gene is indicated by the presence of a blue pigment in the plant tissue.

Table 4.1. The transgenic phenotypes of individual tobacco plants transformed with pJIT119, and of control untransformed tobacco plants.

Plant code	Kanamycin resistance	Asulam resistance	GUS expression
2	+	+	-
3	+	-	-
4	+	-	-
5	+	+	+
6	+	-	-
7	+	-	-
8	+	-	-
9	+	+	+
10	+	-	+
11	+	-	-
Untransformed TL33	-	-	-
Untransformed J6	-	-	-
Untransformed Samsun	-	-	

- = lack of expression of the trait.

+ = presence of the trait.

medium containing asulam. This investigation confirmed there was a difference in growth rate between individual transgenic tobacco plants. The data computed from the image analysis experiment is shown in Table 4.2. A statistical analysis of the data showed that the growth rates were not significantly different. However, useful trends in the response of these samples from each plant can be seen. A graph was drawn from the image analysis data contained in Table 4.2. This graph showed the growth response of a transgenic callus line derived from each transgenic tobacco plant to the herbicide asulam (Fig. 4.6).

The graphic representation of the image analysis data showed that the difference in size (t_1 - t_o) and measured in mm^2 of pieces of callus (n=8) from transgenic tobacco plants 2, 5 and 9, cultured on tissue culture medium containing 50 - 400 mg/l asulam was substantial at all concentrations of asulam (Fig. 4.6). This indicated that these samples of transgenic callus lines were able to proliferate under these conditions, even tolerating 400 mg/l asulam in the medium. These callus lines clearly contained a functional *sul* I gene which enabled them to survive at all tested levels of asulam in the medium. The capacity of the *sul* I gene to confer resistance to the herbicide asulam declined at the highest asulam concentration tested. At an asulam concentration of 400 mg/l, the callus samples from plants 2, 5 and 9 did not increase in size to the same extent as callus samples cultured at lower asulam concentrations (50 mg/l, 100 mg/l and 200 mg/l).

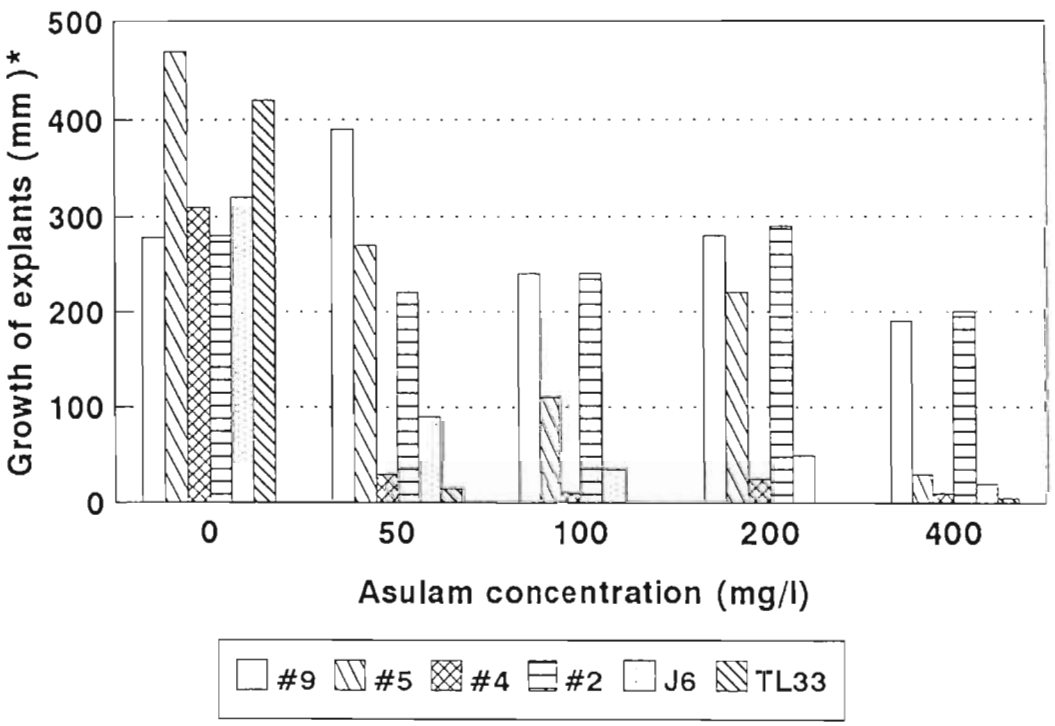
The graphic representation of the image analysis data showed that the samples from a callus line derived from transgenic tobacco plant 4 failed to thrive on tissue culture medium containing asulam (50 - 400 mg/l) (Fig. 4.6). The *sul* I gene was clearly not fully expressed in this plant, although the plant was transgenic for *nptII*. The graphic representation of the image analysis data also showed that the untransformed callus lines from non-transgenic tobacco plants did not increase significantly in size on tissue culture medium containing 50 - 400 mg/l asulam when compared to transgenic callus lines. However, the cultivar J6 was more tolerant to asulam than untransformed TL33.

4.3.3.2 Neomycin phosphotransferase assay. Both assay methods for the assay

Table 4.2. An *in vitro* plant growth experiment to determine the resistance of transgenic tobacco to asulam[a].

Mean change in size of callus samples (t_1 - t_0, mm^2)[b]					
	Asulam concentration[c] (mg/l)				
Plant	O mg/l	50 mg/l	100 mg/l	200 mg/l	400 mg/l
9	277.5	386.9	239.3	283.6	188.5
5	467.6	269.1	113.8	219.9	27.4
4	311.6	26.2	11.2	23.6	7.5
2	282.0	216.1	243.8	288.3	196.7
J6	319.0	86.9	34.0	53.3	18.5
TL33	421.2	14.4	-1.6	-15.7	4.1

a Data collected using computerized image analysis
b n = 8
c Asulam was included in the tissue culture medium.



* Mean of eight values

Fig. 4.6. The effect of asulam concentration on the growth of transgenic and non-transgenic tobacco plants.

of NPTII activity (Cabanes-Bastos *et al.*,1989; Reiss, 1984) were carried out on extracts of transgenic tobacco tissue, non-transgenic tobacco tissue and a bacterial strain, *E.coli*(pGS9), expressing the *nptII* gene. Only the thin layer chromatography (TLC) method of Cabanes-Bastos *et al.* (1989) was sensitive enough to detecte NPTII activity in both transgenic plant extracts and in the bacterial extract (Table 4.3).

The ndPAGE method of Reiss *et al.* (1984) was especially unsuccessful in detecting NPTII activity in plant extracts. Using the Reiss *et al.* (1984) method, this enzyme could only be detected in bacterial extracts. The bacterial protein extract contained approximately 21.7 μg of bacterial protein when assayed. Even though this amount of protein or more than the recommended amount of protein (5 - 10 μg protein) was used in the assay, there was no detection of radioactive kanamycin phosphate in the plant samples. In Table 4.3, a summary of these results is presented. The transgenic plant extracts contained 9.0 μg and 57 μg of protein, but still the NPTII could not be detected in these extracts. The autoradiographic results of the Reiss *et al.* (1984) assay for NPTII activity are shown in Fig. 4.7. It must be noted that other compounds in the plant and bacterial extracts become radioactively phosphorylated during the assay. These compounds separate out from the radioactively phosphorylated kanamycin on the ndPAGE gel.

Using the TLC method of Cabanes-Bostos *et al.*, 1989), NPTII activity could be detected in very small plant and bacterial samples (Fig. 4.8). In the transgenic plant samples, protein amounts of both 0.33 μg and 4.78 μg resulted in NPTII detection (Table 4.3). In the bacterial sample, detection occurred at 4.78 μg. The published amount of protein that results in NPTII detection using this method is in the range 0.5 pg - 500 ng (Cabanes-Bastos *et al.*, 1989). Other compounds in the plant and bacterial extracts also become phosphorylated during the assay, but separate from radioactively phosphorylated kanamycin during thin layer chromatography.

The sensitivity of the TLC method of Cabanes-Bastos *et al.* (1989) and the fact that the method required less radioactive ATP make it the method of choice to detect and quantify NPTII activity in plants.

Table 4.3. The sensitivity of two assay methods to detect neomycin phosphotransferane (NPTII) activity in bacterial and transgenic plant extracts.

Amount of protein (µg total protein) loaded per track				
Sample	**ndPAGE assay (Reiss *et al.*, 1984)[a]**		**TLC assay (Cabanes-Bastos *et al.*,1989)[b]**	
	µg Protein	**Detection**	**µg Protein**	**Detection**
Transformed tobacco leaf	57.0 µg	-	0.198 µg	+
Transformed tobacco callus	9.0 µg	-	0.33 µg	+
E.coli (pGS9)	21.7 µg	+	4.78 µg	+

[a]5 - 10 µg of protein per track reported to enable detection of NPTII (Reiss *al.*, 1984).

[b]0.5 pg to 500 ng of protein per track reported to enable NPTII detection (Cabanes-Bastos, 1989).

+ = NPTII activity detected in this study.

- = no NPTII activity detected in this study.

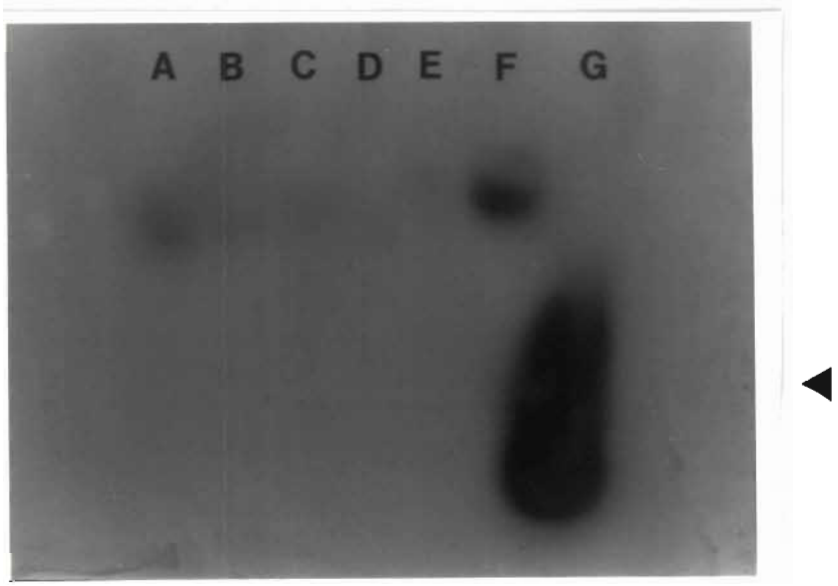


Figure 4.7. Autoradiograph of extracts of plant and bacterial extracts assayed for NPTII activity (Reiss *et al.*, 1984). Lanes A - C, transgenic tobacco extracts. Lanes D - F, non-transgenic tobacco extracts. Lane G, bacterial extract containing NPTII activity. The NPTII activity, were present, is shown by the presence of [α - ^{32}P] labelled kanamycin phosphate («). Other radioactively phosphorylated compounds are also present.

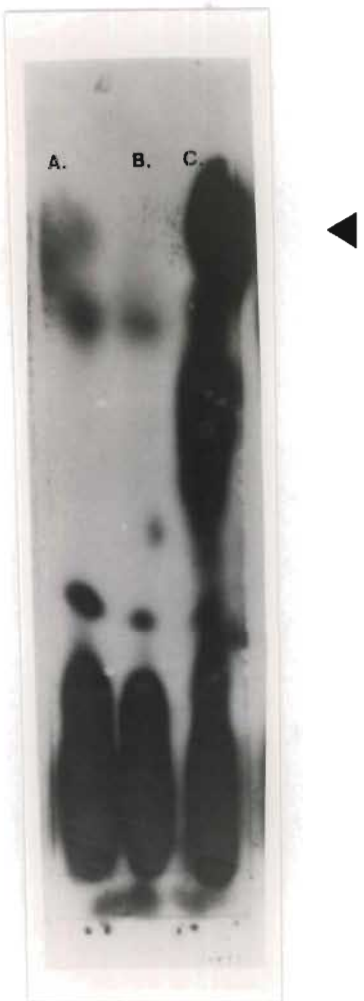


Fig.4.8. Autoradiograph of plant and bacterial extracts assayed for NPTII activity (Cabanes-Bastos *et al.*, 1989). Lane A, transgenic tobacco extract. Lane B, non-transgenic tobacco extract. Lane C, bacterial extract with NPTII activity. NPTII activity is indicated by the presence of [α - ^{32}P] labelled kanamycin phosphate («). Other radioactively phosphorylated compounds are also present.

4.3.3.3 Western blotting. The Western blot method of Scott *et al.* (1988) detected the presence of the NPTII protein in a bacterial sample containing 217 μg protein. No NPTII could be detected in each of two protein samples extracted from transgenic tobacco plants, and containing 9.0 μg and 57.0 μg protein respectively (data not shown). The reported amount of protein that can be assayed by the Western blot method to detect a protein was 50ng (Scott *et al.*, 1988).

4.4 Discussion.

This study confirmed that certain tobacco plants resulting from *Agrobacterium*-mediated leaf disc transformation experiments with the binary vector, pJIT119, were fully transgenic. Plants which had "escaped" selection were eliminated by identifying those plants which were bleached or did not root on medium containing kanamycin. Before the expense of greenhouse and field trials are undertaken to test transgenic plants, the most promising transgenic plants must be identified in the laboratory. This detailed study of the expression of foreign genes in transgenic tobacco plants identified those plants which would be suitable for greenhouse and field testing, and which could be considered for inclusion in a breeding programme for tobacco improvement.

The triplet of foreign genes, *sul* I, *nptII* and *uidA* which is transferred into plants from plasmid pJIT119 using *Agrobacterium*-mediated transformation, provided a useful system for the study of gene expression in a small group of ten transgenic tobacco plants.

Transgenicity of ten tobacco plants was confirmed through detecting the expression of the *nptII* gene. The study showed that tobacco plants 2 - 11 were able to grow normally on tissue culture medium containing kanamycin, producing green, unbleached leaves. In addition, the plantlets were able to root on medium containing kanamycin. Leaf material from each plant also produced callus when cultured on callus inducing medium containing kanamycin. Normal plant growth and colour, as well as proliferation of callus under these conditions indicated the presence of a functional *nptII* gene in the plants. The *nptII* gene conferred resistance to the antibiotic kanamycin. Plants which had "escaped" kanamycin selection were eliminated from further experimentation.

Assays were also used to study the expression of foreign genes *sul* I and *uidA*. The *in vitro* tolerance of transgenic tobacco plants to asulam was used to confirm the presence and expression of the *sul* I gene. The growth of callus lines produced from plants 2 - 11 cultured on medium containing asulam, as well as shoot induction from

leaf explants cultured on medium containing asulam indicated that several of the transgenic tobacco plants were resistant to asulam. The histochemical assay for ß-glucuronidase (GUS) was employed to confirm the presence and expression of the *uidA* gene. Several of the plants expressed the *uidA* gene. These assays determined the transgenic phenotype of each tobacco plant derived from South African commercial tobacco varieties.

Interestingly, not all of the transgenic tobacco plants which expressed the *nptII* gene were resistant to asulam or showed expression of the *uidA* gene. It was originally assumed that each plant would express all three foreign genes from pJIT119. A high level of inactivation of the foreign genes not maintained under selection in transgenic tobacco plants was, therefore, indicated by these results. Gene inactivation occurred in both the *sul* I and *uidA* genes which were not maintained under selection during experimentation. The *sul* I gene in transgenic material was not maintained under constant asulam selection during the leaf disc transformation experiment and subsequent culture of the transgenic plants. The ß-glucuronidase reporter gene cannot be directly selected for.

The most inconvenient aspect of using kanamycin as the sole means of selection in *Agrobacterium*-mediated transformation was that defects in the other foreign genes co-transferred with the *nptII* gene remained undetected during the early stages of experimentation. The plants which were selected on the basis of an active *nptII* gene were subsequently found to contain defective *uidA* and/or *sul* I genes. It was found that only three out of ten transgenic tobacco plants expressed all three foreign genes transferred into them from pJIT119. If possible, transgenic plants should be screened for the presence and expression of all co-transferred foreign genes as early as possible. Those plants which do not express the *nptII* gene may still be valuable plants with regard to the other foreign genes which they express. It is highly likely that "escape" plants contained fully functional ß-glucuronidase and/or *sul* I genes.

The large number of transgenic plants which do not contain or express the desired foreign genes does present a problem in the genetic engineering of plants for traits

other than selectable markers (Blake *et al.*, 1991). This is a particular problem when the important foreign gene cannot be selected for. The conventional approach that has been taken to overcome this difficulty is to attach a selectable marker gene (such as *nptII*), to a second gene of interest which may be a gene for virus resistance, improved starch quality, longer shelf life of fruit, or improved cotton fibre quality using recombinant DNA techniques. A selection procedure based on selection for the marker gene is applied to identify the transgenic shoots or plants expressing the selectable marker gene. It is hoped that these transgenic plants will also express the non-selectable gene of interest. Assays for the expression of the second gene may be difficult or impossible early on in the development of the transformed plant, leading to a reliance on the expression of a selectable marker gene to indicate a fully transgenic plant. This reliance leads to a failure to detect "escapes" in the non-selected genes early on.

The use of PCR to monitor multiple foreign gene insertions at an early stage in plant transformation experiments has been proposed (Blake *et al.*, 1991). Because PCR requires a very small DNA template, tiny samples of transgenic plants can be assayed at a very early stage. This will give information about the presence of foreign genes, but not whether they are expressed. A technique like Western blotting would also be of use to detect the translation products of foreign genes that cannot be selected for (Scott *et al.,* 1988).

To overcome the problem of a high percentage of transgenic plants selected in this manner not expressing the desired gene, a very large number of individual transgenic plants must be generated, propagated and screened. Once a large group of plants expressing the desired non-selectable gene has been identified, studies to determine quantitative differences between foreign gene expression in these plants can be undertaken. This would enable the identification of those transgenic plants expressing the foreign gene maximally.

Of the NPTII assay methods under trial in this study, only the method of Cabanes-Bastos *et al.* (1989) proved to be sensitive enough to detect NPTII activity in extracts of plant material. Both the radioactive assay methods of Reiss *et al.* (1984) and

Cabanes-Bastos *et al.*(1989), as well as Western blotting, could detect NPTII activity in bacterial extracts. The radioactive assays for NPTII were both complex, requiring the use of large amount of radioactive material and specialized facilities for the handling of radioactivity. The Western blotting method, which would be less hazardous to carry out, was not sensitive enough to detect NPTII in plant extracts. Because of the importance of the *nptII* as a marker gene, new methods have been published which supercede previous methods. The method of Sahasradbudhe and Roy (1990) is claimed to be more sensitive and easier to perform that the methods of Cabanes-Bastos *et al.*(1989) and Reiss *et al.* (1984). An enzyme linked immunosorbent assay (ELISA) method is also safe and convenient (Nagel *et al.*, 1982). However, the use of radioactive NPTII assays to confirm transformation and quantify foreign gene expression has largely been superceded by the GUS (B-glucuronidase) assay. The GUS assay is less hazardous and is more rapid to perform.

A study using computerized image analysis was undertaken to measure exact differences in the tolerance of the individual transgenic plants to the herbicide asulam. The time required to use image analysis to process a statistically significant number of samples was identified as a significant disadvantage of this technique. Lengthy computations are carried out by the image analyzer for each sample, and when many samples have to be analyzed, image analysis is not practical. Image analysis, however, did accurately measure the comparative growth of callus from individual transgenic tobacco plants and this information could be graphically displayed. The data confirmed previous results where certain individual plants (plants 2, 5 and 9) had been shown to express high levels of asulam resistance.

In this study, transgenic plants 2, 5 and 9 expressed the *sul* I gene efficiently *in vitro* and would therefore be suitable for further assessment to determine their potential as herbicide resistant plants in the greenhouse, and finally in the field.

To conclude, the commercial use of transgenic crop plants requires that transgenic plants are quickly identified following transformation experiments, and plants not expressing foreign genes are discarded. Those plants with maximal foreign gene

expression must be identified in laboratory studies. These plants will undergo further greenhouse and field testing to determine the foreign gene expression under these conditions. Progeny of these plants will also undergo field testing to determine the inheritance and stability of foreign genes over time.

Chapter 5

Analysis of the presence and expression of the *nptII*, *sul* I and *uidA* genes in selected transgenic tobacco plants using the polymerase chain reaction, *in situ* hybridization and the GUS histochemical assay.

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5.0 Summary

A group of ten transgenic tobacco plants showed a high level of foreign gene inactivation when analyzed. Each transgenic plant was therefore unique because of this phenomenon. Foreign gene inactivation in transgenic plants can be due either to whole gene loss occurring during T-DNA transfer and integration, or due to rearrangements or deletions within the genes carrried on the T-DNA (Dunsmuir *et al.*, 1988). The suppression of foreign gene expression by DNA methylation or by the position effect is also common in transgenic plants. To determine which of these factors was responsible for foreign gene inactivation in the tobacco plants, the polymerase chain reaction (PCR), *in situ* hybridization and the GUS histochemical assay were carried out.

The promoter used to control foreign gene activity in transgenic plants is the cauliflower mosaic virus (CaMV) 35S promoter. This promoter has a number of endogenous properties, including a strong tissue specific expression. Using transgenic tobacco plants as a model system, *in situ* hybridization located the tissues and cells where foreign gene expression under the control of the 35S promoter occurred. Promoter activity was concentrated in the vascular region and glandular hairs in these plants.

5.1 Introduction

5.1.1 Inactivation of foreign gene expression in transgenic plants. The most noticeable aspect of a group of ten transgenic tobacco plants obtained from an *Agrobacterium*-mediated leaf disc transformation experiment and assayed for foreign gene expression, was that each plant was unique. The individuality of each transgenic plant was caused by the high rate of foreign gene (*uidA*, *sul* I and *nptII*) inactivation in the transgenic plants. This phenomenon of foreign gene inactivation is common in transgenic plants (Dunsmuir *et al.*, 1988; Deroles and Gardner, 1988; Blake *et al.*, 1991). In this present study, it was found that the expression of either one or two of the three T-DNA encoded foreign genes from pJIT119 was permanently absent in the plants. The foreign gene inactivation was maintained throughout the life cycle of each plant, including during *in vitro* manipulation. The foreign gene inactivation was also inherited by the progeny.

Inactivation of foreign gene expression in transgenic plants can be due either to actual foreign gene loss (Blake *et al.*, 1991), or to inversions, truncations or deletions which disable the foreign genes during or subsequent to the original T-DNA integration event (Gheysen *et al.*, 1990; Mayerhofer *et al.*, 1991). Aberrant forms of T-DNA insertions are found in transformed plants with varying frequencies (1% - 40%), most commonly showing a deletion at one or both ends of the T-DNA (Gheysen *et al.*, 1991). Deletions of up to 50 bp at the left border of T-DNA frequently occur (Hensgens *et al.*, 1992). It is not known exactly how and when these truncated T-DNAs are formed, whether during synthesis, transfer or ingetration of the T-DNA. One suggestion (Van Lijsebettens *et al.*, 1986) is that truncated T-DNAs can arise from the recognition of "pseudoborders" during T-strand synthesis in the bacterium, resulting in shortened T-strands. This, however, occurs at a very low frequency and other mechanisms must also be responsible for a random distribution of endpoints in the T-DNA resulting in truncations (Herman *et al.*, 1990). These mechanisms could be: (i) the use of random nicks for the synthesis of shortened T-strands, (ii) digestion of the T-DNA ends by nucleases prior to integration, (iii) breakage of the T-DNA during the transfer process (i.e. in the bacterium or in the plant), and (iv) rearrangements generated during integration (Herman *et al.*, 1990; Gheysen *et al.*, 1990; Mayerhofer *et al.*, 1991).

Herman *et al.* (1990) favour the theory that shortened T-DNA insertions are generated by breakages during the transfer or the integration process, probably after synthesis of a normal T-DNA intermediate. Their research showed that truncation occurred with equal frequency in different DNA sequences and at different distances from the right border, indicating that truncation is a random process and is not sequence dependent. Truncations were also found to be equally distributed over the right and left border regions of the T-DNA (Herman *et al.*, 1990).

Foreign gene expression level variability in transgenic plants can also be explained in many cases by "position" effects. Expression levels of introduced genes are thought to be directly influenced by the local structure and modification pattern of the chromatin region at or near to the site (position) of T-DNA integration (Peach and Velten, 1992; Breyne *et al.*, 1992). The higher order of the chromatic structure of the region surrounding the integration site might also influence gene expression. Eukaryotic DNA is also thought to be organized in looped domains, which function as independent genomic units (Eissenberg and Elgin, 1991). The specific loop in which the T-DNA has inserted may partially determine the overall expression level of these genes. Foreign gene expression can also be influenced directly by neighbouring plant regulatory sequences (enhancers or silencers) (Horsch *et al.*, 1988; Dunsmuir *et al.*, 1988; Peach and Velten, 1991; Breyne *et al.*, 1992).

DNA methylation can also be a possible cause of the lack of foreign gene expression in transgenic plants (Deroles and Gardner, 1988; Renckens *et al.*, 1992; Ottaviani *et al.*, 1993; Palmgren *et al.*, 1993). Methylation of DNA is a widespread modification event in eukaryotic cells, and the level of DNA methylation is thought be be involved in the regulation of gene expression. In plants, up to 30% of cytosines are methylated. Following T-DNA entry into plant cells and integration into the genome, T-DNA genes can be methylated and inactivated (Ottaviani *et al.*, 1993; Palmgren *et al.*, 1993)). *Agrobacterium* also methylates 0.5% of all cytosines, and transgenes might be methylated already before entering the cell and already potentially inactivated (Palmgren *et al.*, 1993). Selective repression of cointroduced genes by DNA methylation can occur. For example, the *uidA* gene appears to be more sensitive to methylation than the *nptII* gene (Bochardt *et al.*, 1992; Ottaviani *et al.*, 1993). When

DNA methylation occurs, there is no mRNA produced from these methylated genes (Ottaviani *et al.*, 1993). The methylation of T-DNA can be maintained unchanged during mitosis and different phases of *in vitro* culture, as well as during meoisis (Bochard *et al.*, 1992). Genes inactivated by methylation can be reactivated following 5-azacytidine (azaC) treatment (Renckens *et al.*, 1992; Mandal *et al.*, 1993; Palmgren *et al.*, 1993).

To summarize, the possible causes of the failure of foreign genes to express in transgenic plants are the following: a complete loss of the T-DNA from the transgenic plant (Blake *et al.*, 1991); the loss of a single foreign gene from the transgenic plant; the inactivation of a single foreign gene through DNA rearrangements or deletions (Gheysen *et al.*, 1990); the inactivation of foreign genes through the "position" effect (Peach and Velten, 1991) and the loss of foreign gene expression due to methylation of foreign genes (Palmgren *et al.*, 1993)

A number of assumptions can be made to confirm or refute each particular form of foreign gene inactivation in a particular group of transgenic plants. To confirm complete loss of the T-DNA, the T-DNA would be undetectable in the plant genome using Southern blot analysis or PCR. The deletion of a single foreign gene from the T-DNA integrated into the plant genome could also be confirmed through Southern blot analysis and PCR. mRNA would not be transcribed from a gene suspected of being deleted, and hence *in situ* hybridization, together with a negative PCR or Southern blot analysis result could confirm that a gene had been deleted. Rearrangements within single defective foreign genes could be inferred from observations that while the foreign DNA was present and transcription occurred, the final gene product might be defective, but not absent.

The failure of foreign genes to express at certain stages of plant growth could be linked to the "position" effect. Also, were the "position" effect responsible for foreign gene inactivation, foreign gene expression might vary at different stages of the plant life cycle. If a "position" effect inactivation had occurred, foreign DNA would be detectable using Southern hybridization and PCR, even during an inactive phase, but no mRNA would be transcribed during the inactive phase. DNA methylation can

impose selective or total foreign gene inactivation. The specific foreign DNA, however, is still present and can be detected using Southern hybridization and PCR. No mRNA is produced from methylated genes as they are not transcribed. The pattern of foreign gene inactivation following methylation is maintained throughout the life cycle of the plant, but gene activity can be restored using 5-azacytidine (Palmgren *et al.*, 1993).

In those transgenic tobacco plants which did not express several of the foreign genes encoded by the T-DNA of pJIT119, it was thought to be important to determine whether the inactive foreign genes were still present in the plant genome, and whether foreign mRNA was being transcribed from these genes. The polymerase chain reaction was used to confirm the presence of DNA belonging to each foreign gene in the transgenic tobacco plants. *In situ* hybridization was used to show the presence of specific foreign mRNA types. The use of *in situ* hybridization for the study of foreign gene inactivation in transgenic plants has not been reported in the scientific literature.

In situ hybridization also has a more wide-spread use. The technique of *in situ* hybridization can be used to locate endogenous gene transcription within tissues through specific mRNA detection. Foreign gene expression can also be detected using *in situ* hybridization.

5.1.2 The polymerase chain reaction (PCR). The polymerase chain reaction is a powerful technique used in many areas of biological research. The technique allows *in vitro* amplification of specific DNA sequences from almost undetectable quantities of target DNA (Saiki, 1989). Using short oligonucleotide primers and a heat resistant DNA polymerase, it is possible to cycle minute amounts of target DNA through a series of denaturation, reannealing and polymerisation steps. This results in a significant amplification of the DNA sequence situated between the two primers. PCR is very useful for screening transformed plants for integration of foreign DNA and is quicker and more effective than Southern blot analysis. Techniques have also been developed whereby small leaf or root pieces are placed directly in the PCR reaction mixture for amplification of foreign genes *in situ* (Berthomieu and Meyer, 1991).

5.1.3 *In situ* hybridization. *In situ* hybridization is a labelled nucleic acid technique,

whereby radioactively or a non-readioactively labelled nucleic acids are used as probes to locate complemetary sequences on chromosomes or within tissues. *In situ* hybridization was originally developed for animal tissues to determine the location of a particular mRNA or to map chromosomal positions of cloned DNA (Lichter *et al.*, 1991; Narayanswami and Hamkalo, 1991). More recently, *in situ* hybridization has been applied to the localization of specific RNAs in plant tissues (Koltunow *et al.*, 1990; Olmedilla *et al.*, 1991; Melzer *et al.*, 1990), as well as localizing genes on plant chromosomes (Huang *et al.*, 1989; Griffor *et al.*, 1991) and for imaging viroids in plant tissues (Harders *et al.*, 1989).

In situ hybridization is very useful for detecting and locating foreign gene expression in transgenic plant tissue. When foreign genes are expressed following the integration of T-DNA into the plant genome, large amounts of foreign specific mRNA accumulate in transgenic tissues and cells. *In situ* hybridization can be employed to locate this foreign mRNA. *In situ* hybridization was carried out in this present study to identify and locate foreign mRNA in transgenic plants made from South African tobacco cultivars.

The main steps of *in situ* hybridization include choice of nucleic acid labelling system (radioactive or non-radioactive), DNA probe identification, isolation and labelling, sectioning and fixing the plant tissue, prehybridization treatments including protease treatment and acetylation, hybridization of the probes to the tissue sections, post-hybridization washes, autoradiography, photography and data analysis (Ausubel *et al.*, 1988; Raikhel *et al.*, 1989).

Both RNA or double stranded DNA probes can be used for *in situ* hybridization. For non-autoradiographic *in situ* hybridization, nucleic acid probes can be labelled with fluorescent (Griffor *et al.*, 1991), biotinylated (Harders *et al.*, 1989) or digoxigenin (Boehringer Mannheim) labels. For autoradiographic *in situ* hybridization of mRNA, ^{3}H (Huang *et al.*, 1989) or ^{35}S (Olmedilla *et al.*, 1991) labels are generally used. Although most methods of *in situ* hybridization are designed for the detection of gene location at the light microscope level, methods have also been developed for plant tissues to be examined at the electron microscope level (Meyerowitz, 1987). A simplified "tissue squash" method of *in situ* hybridization has also been devised which enables the

monitoring of organ and tissue specific mRNA expression in seedlings without the extensive tissue fixing steps normally required for *in situ* hybridization (McClure and Guilfoyle, 1989).

If autoradiography is to be used to detect specific mRNA species, radioactive isotopes (^{3}H, ^{35}S and ^{32}P) can be used to label the DNA probes. Each of these isotopes has certain advantages and disadvantages. ^{3}H is a weak beta emitter and when used with emulsion autoradiography can yield single cell resolution. However, the exposure time is long (several weeks). ^{32}P is a strong beta emitter, and although film autoradiography can be used, the long path length of each particle makes fine tissue and cell resolution impossible. A compromise is to use ^{35}S which results in adequate cellular resolution with short exposure times when using emulsion autoradiography (Ausubel *et al.*, 1988). DNA or RNA probes can be labelled with ^{35}S using nick-translation or random primed kits.

Several other assay methods are available to detect and quantify cellular mRNA, but *in situ* hybridization is the only method which enables the direct location of mRNA in cells or tissues (Raikhel *et al.*, 1989). *In situ* hybridization is not quantitative, but semi-quantitative information can be obtained because differences in RNA concentrations are accurately reflected in relative grain densities (Cox and Goldberg,1988).

5.1.4 Tissue specificity of foreign gene expression under cauliflower mosaic virus (CaMV) 35S promoter control in transgenic tobacco plants. The tissue specific activity of the cauliflower mosaic virus (CaMV) 35S promoter is thought to occur through the interaction of a series of discrete *cis* promoter elements called domains with *trans* acting protein factors present in different types of plant cells (Benfey *et al.*, 1989; Benfey *et al.*, 1990). In the CaMV 35S promoter, there are two domains and five subdomains, and these multiple domains can function either independently or synergistically to activate the 35S promoter in a developmental and tissue-specific manner. Using ß-glucuronidase activity in transgenic plants as the assay system, two models for the combinatorial code of the 35S promoter have been proposed following an analysis of expression patterns of the *uidA* gene under the control of the CaMV 35S promoter throughout plant development. The tissue specific

expression patterns conferred by subdomains of the 35S enhancer in transgenic tobacco have been described in detail (Benfey *et al.*, 1990).

The first model explains how cooperative interactions between heterologous factors enable binding to *cis*-elements when both factors are in low concentrations in the cell. This type of model predicts that a cell that contains a high concentration of an active *trans*-factor should be able to activate transcription from genes that contain a binding site for the factor. In the situation when active factor concentration is low, binding to the *cis*-element would not occur unless there was a cooperative interaction with another factor. An alternative model postulates that synergism is mediated by an interaction between bound *trans*-factors and a target factor. In this model, the target factor must interact with more than one bound factor at a time. Synergism arises from the necessity to have a minimum number of sites filled to obtain a productive interaction (Benfey *et al.*, 1990).

In a study of the CaMV 35S promoter domains in transgenic tobacco, activity of this promoter can be found primarily associated with the phloem elements in stems and leaves, as well as with the stem and leaf trichomes, and in root phloem (Benfey *et al.*, 1990). Tissue specificity of the CaMV 35S promoter activity has also been shown in transgenic monocots (rice). In rice, activity of the 35S promoter was primarily localized at or around the vascular tissue in leaf, root and flower embryo (Terada and Shimamoto, 1990; Battraw and Hall, 1990).

5.1.5 Anatomy of the tobacco stem, leaf midrib and glandular hair. The tobacco stem has a cylindrical vascular system (siphonostele), surrounded by cortex to the outside, and pith to the inside. In tobacco, the arrangement of the siphonostele is amphiphloic, in which the phloem surrounds the xylem both externally and internally (Fahn, 1990). The phloem and xylem form a continuous layer in the tobacco stem. The starch sheath appears as a continual single cell layer outside the external phloem. The vascular system of the tobacco leaf midrib and petiole is a crescent-shaped structure, with internal phloem located adaxially to the central xylem, and the external phloem located abaxially to the xylem.

Trichomes (hairs) cover most surfaces of plants. In plants, trichomes may be simple hairs which deter herbivores or may be more specialized tissues (glandular trichomes) whose principal function(s) may be to produce pest- or pollinator interactive chemicals. In glandular trichomes, large quantities of metabolic products accumulate in structures designed for this purpose (Wagner, 1991). In tobacco, the trichomes which cover the surface of leaves and stems are of the glandular type. They consist of a stalk supporting a dark coloured multicellular gland (Esau, 1965). The surface of tobacco plants often accumulate small insects which have become trapped by the secretions of the glandular hairs. Systems like this may comprise a physiochemical defense mechanism in plants. Nicotine-related alkaloids are significant pest-interactive components of *Nicotiana* trichome exudates (Wagner, 1991).

5.2 Materials and Methods

5.2.1 Primer construction for the polymerase chain reaction. Oligonucleotide primers (12-mers for the *sul* I and *nptII* genes, and 21-mers for *uidA* gene) to be used as primers for the PCR amplification of the *uidA*, *sul* I and *nptII* genes in transgenic tobacco genomic DNA were synthesized by the Biochemistry Department of the University of Cape Town.

Oligonucleotide primers used in the polymerase chain reaction to detect the *uidA* gene in the genomic DNA of transgenic tobacco plants were as follows. *UidA* primer 1: GGT GGG AAA GCG CGT TAC AAG; *uidA* primer 2: GTT TAC GCG TTG CTT CCG CCA. These primers included positions 400 to 420 and 1599 to 1597 of the *Uid*A gene (Hamill *et al.*, 1991). The expected size of DNA amplification product was 1.2 kb. The size of the *uidA* gene in pJIT119 was 1.75 kb (estimated from a key provided in the Plant Gene Toolkit manual).

Oligonucleotide primers used in the polymerase chain reaction to detect the *sul* I gene in the genomic DNA of transgenic tobacco plants were as follows. *Sul* I primer 1: CGA ACA CCG TCA; *sul* I primer 2: GGC ATG ATC TAA These primers included positions 668 to 680 and 1480 to 1492 of the *sul* I gene. The expected size of the PCR amplification product is 575 bp. The size of the *sul* I gene in pJIT119 is estimated from a key in the Plant Gene Toolkit manual to be 650 bp

The oligonucleotide primers used in the polymerase chain reaction to detect the *nptII* gene in the genomic DNA of transgenic tobacco plants are as follows: *nptII* primer 1: GAG GCT ATT CGG; *nptII* primer 2: GGC GAT ACC GTA. These primers correspond to positions 201 to 213 and 867 to 879 of the gene. The expected size of amplification product is 700 bp (Hamill *et al.*, 1991).

5.2.2 Preparation of transgenic plant material for foreign DNA detection using the polymerase chain raction (PCR). The transgenic plant material used for PCR analysis was obtained from selfed (S_1) progeny seedlings from the self-pollination of the original transgenic (T_0) plants 2 - 11. The original plants 2 - 11 were no longer

available at the time of carrying out the PCR experiments as they had been sacrificed in earlier experiments.

The transgenic seeds were surface sterilized in 33% bleach (= 1.2% sodium hypochlorite) for twenty minutes, and rinsed well in sterile water. The transgenic progeny seedlings were germinated *in vitro* on MS (Murashige and Skoog, 1962) medium containing kanamycin (100 mg/l) to segregate the kan^r and kan^s progeny. Genomic DNA from only the kan^r progeny from each transgenic plant was extracted and analyzed using PCR. This to make certain that the segregants contained the foreign genes, which co-segregated with the *nptII* gene. Kanamycin sensitive segregants contain no foreign genes in examples of transgenic plants with a single T-DNA insertion. In this study, transgenic tobacco plants 2, 3, 5, 7 and 11 contained single T-DNA insertions (Chapter 6). Plant 9 contained a double T-DNA insertions with gene inactivations in the second T-DNA insert (Chapter 6). However, kan^s progeny from plants 2 and 9 were also analyzed using PCR. The kan^s progeny of plant 2 were used as negative controls containing no foreign genes. The kan^s progeny of plant 9 were interesting because of the double T-DNA insertion with inactivated *nptII* and *sul* I genes (Chapter 6).

5.2.3 Plant genomic DNA extraction for PCR detection of foreign DNA. Total genomic DNA was extracted from transgenic tobacco seedlings using a SDS method from Draper *et al*. (1988). The final DNA pellet obtained for each sample was extracted twice with a 24:1:24 mixture of TE saturated phenol, isoamyl alcohol and chloroform. To precipitate the DNA, 300 μl chilled isopropanol and 50 μl 3M sodium acetate was added to 500 μl of DNA in TE buffer. The mixture was chilled for 30 minutes at -20°C before being pelleted at 12 000 rpm. The final DNA pellet was resuspended in 500 μl TE buffer. Bacterial plasmid DNA (pJIT119) used as a control for the PCR process was prepared according to Birnboim and Doly (1979). The DNA concentration of the preparations was determined by electrophoresis and comparison with lamdba DNA of known amounts. The DNA was dialyzed against sterile distilled water. Phenol extraction and subsequent dialysis was essential for successful PCR amplification. Aliquots of the preparations were dialyzed against sterile distilled water for four hours. The dialysis was carried out by placing 10 μl of the DNA solution on a Millipore disc filter (0.025 μm pore size) floated on the surface of

The droplets of DNA were collected after four hours, the volume measured and the final DNA concentration determined from the dilution factor.

5.2.4 Optimization of the polymerase chain reaction to detect foreign DNA in transgenic plant genomic DNA. To optimize the polymerase chain reaction for tobacco and bacterial DNA, the DNA concentration, the number of units of *Taq* polymerase (Promega), the $MgCl_2$ concentration, and the annealing temperatures was experimentally determined for each primer pair. After optimization, the PCR reactions using the *sul* I and *nptII* 12-mer primers were carried out at an annealing temperature of 50 °C and for the 21-mer *uidA* primer pairs, an annealing temperature of 60 °C was used. In all cases, the reaction mixture consisted of 50 - 100 ng of plant genomic DNA or bacterial DNA, 200 μM of each dNTP (Boehringer Mannheim), 1 μM of each primer, 2.0 units of *Taq* polymerase in PCR buffer (50 mM KCl, 10 mM Tris/HCl pH9.0 at 25 °C, 1.5mM $MgCl_2$), overlaid with sterile paraffin oil. The samples were then subjected to amplification in a Hybaid OmniGene thermal cycler. Denaturation for 5 min at 94 °C was followed by 30 cycles of amplification (1 min at 94 °C, 2 min at 60 °C (*uidA* primer) or 50 °C (*sul* I, *nptII*), 2 min at 72 °C). After this, 5 μl of gel-loading buffer containing 0.25% bromophenol blue and 40% sucrose in water (Sambrook *et al.*, 1989) was added to each reaction mix. The PCR reaction was loaded directly onto a 1.5% agarose (Seaplaque) gel for electrophoretic analysis. A lambda DNA digested with *pst*I was used as the molecular weight marker. The gels were stained in ethidium bromide and viewed under ultraviolet light. Results were recorded photographically.

5.2.5 Tissue fixation and sectioning for *In situ* hybridization. To prepare the transgenic tobacco leaf midribs and stems for *in situ* hybridization, hand cut sections were made using razor blades of plants 2, 3, 5, 6, 9 and 10, as well as of untransformed tobacco (cv. J6). Meristems and leaves were also sectioned, but, it was not possible to obtain good thin leaf sections of this material by hand. The sections were processed in 25-well Greiner boxes. The tissue sections were placed in the wells, and the solvents or fixatives added and withdrawn using a syringe and needle. Similarly, the prehybridization, hybridization and post hybridization steps were carried out in these boxes. *In situ* hybridization was carried out according to Ausubel *et al.* (1989).

Fixation of tobacco tissue sections was in 4% paraformaldehyde, followed by dehydration of the sections in an ethanol series (30%, 60%, 80%, 95% and 100%). To continue the tissue processing for *in situ* hybridization, the sections were acetyleated in freshly prepared triethanolamine (TEA) buffer, rinsed and dehydrated by passaging through another ethanol series (30%, 60%, 80%, 95% and 100%). Hybridization was performed immediately after this step. The radioactively labelled DNA probes were resuspended in 2 parts hybridization mix B (Ausubel *et al.*, 1989) and 2 parts deionized formamide, with 1 part 50% dextran sulfate added. The probe was boiled for two minutes and then chilled on ice. Using the tip of a Pasteur pipet, the probes were distributed over the tissue sections. Incubation to achieve hybridization was for four hours at 37 °C. After the hybridization, extensive washing with prewarmed DNA wash solution was carried out (Ausubel *et al.*, 1989). Following washing, the tissue was dehydrated through an ethanol series and air dried. After prehybridization, hybridization and post-hybridization, the tissue sections were adhered to glass microscope slides coated with Haupt's adhesive and allowed to dry. Haupt's adhesive is made from 1 g gelatine powder dissolved in 100 ml distilled water at 30 - 40 °C. Two grams phenol crystals and 15 ml glycerol were added to the dissolved gelatine and filtered. Tissue sections were dipped in paraformaldehyde and placed on the coated slides to adhere, and were then dried. Hybridization was detected by autoradiography (Ausubel *et al.*, 1989).

5.2.6 Probe construction and labelling for *in situ* hybridization. Radioactively labelled double stranded DNA probes were prepared from plasmid DNA. A 1.8 kb *uid*A gene probe was prepared from pJIT58/1 DNA digested with *Bam* HI. A 0.5 kb *sul* I gene fragment was cut from pJIT119 using a *Pst* I digest. Following restriction enzyme digestion and DNA electrophoresis, DNA bands were excised from 0.8% seakem agarose gels for radioactive labelling. The method of DNA extraction from agarose was to cut out the appropriate DNA bands and place them in dialysis tubing containing 0.5 X TBE buffer (Sambrook *et al.*, 1989). The dialysis tubing was placed in the buffer tank of the electrophoresis apparatus and electrophoresed out of the agarose at 200 V for 30 minutes . The DNA was precipitated using lithium chloride (Sambrook *et al.*, 1989). The final DNA concentration before labelling was 50 ng/μl.

Radioactively labelled double-stranded DNA probes were synthesized using the Boehringer Mannheim Random Primed method using [^{35}S]dNTPs. In the standard labelling reaction, [^{32}P]dNTP is replaced with two different [^{35}]dNTPs, [^{35}S]dATP and [^{35}S]dCTP. In each reaction, 500 ng of DNA was labelled, and two reactions were performed for each probe to obtain enough labelled DNA for *in situ* hybridization. The activity of the radiolabelled probes was between 1.7 - 2.6 x 10^6 cpm, determined using liquid scintillation counting. The amount of labelled probe required for *in situ* hybridization was 0.2 ug DNA/ml of hybridization mix B (Ausubel *et al.*, 1989) per kilobase of probe. For the *uidA* probe, therefore, 100 ug DNA/ml of hybridization mix B was required, and for the *sul* I probe, 400 ng/ml of hybridization mix B was required (Ausubel *et al.*, 1989).

5.2.7 Autoradiography. Following *in situ* hybridization, a liquid photographic emulsion was applied to the hybridized tobacco sections following extensive post-hybridization washing. Emulsion (LM-1) (Amersham) for light microscopy was used according to the manufacturer's instructions. However, instead of dipping the glass microscope slides into the emulsion to coat the tissue sections, it was more economical to paint the emulsion over the tissue sections attached to the slides. An inexpensive artist's brush was used for this purpose.

The exposure of the emulsion layer to the radioactively probed tissue sections was carried out in a light proof chamber made from a small cardboard box sealed with masking tape. Silica gel was used to maintain dehydrating conditions within the box. The box was wrapped in aluminium foil before being taken into the light for transfer to a cold room for incubation at 4 °C for four hours. All manipulations of the photograhic emulsion and emulsion coated slides were carried out in darkness, or with a red safe light on. The safe light had been additionally shielded with aluminium foil so that the amount of red light was kept to a minimum.

5.2.8 Location of foreign gene (*uid*A) expression in transgenic tobacco material using the GUS histochemical assay and biological stains.

5.2.8.1 Sample preparation. Unfixed hand-cut sections of transgenic tobacco stems

and leaf midribs were used for the GUS histochemical assays. Uncut sections were also stained with toluidine blue and iodine.

Friable callus was prepared from leaf material using the callus induction method outlined in Chapter 3. Leaf material from a S_2 progeny of the original plant 9 (GUS positive) was used. Surface sterilized leaf material was placed on callus inducing MS medium (Chapter 3) and cultured in the dark at 26 ° C for four weeks. Samples of the callus which developed at the leaf edges was assayed for GUS activity using the histochemical method of Jefferson (1987).

The cells of the callus were also stained for viability using 0.01% toluidine blue. Aliquots of callus cells were heat killed by placing them in an eppindorf tube and floating this on the surface of boiling water for fifteen minutes. The heat killed cells were mixed in a 1:5 ratio with non-heat treated cells and stained with 0.01% toluidine blue to confirm that toluidine blue staining could differentiate between living and non-living plant cells.

5.2.8.2 GUS histochemical assay. A standard GUS histochemical assay was carried out on unfixed, hand-cut sections of transgenic stems and leaf midribs (Jefferson, 1987). The GUS histochemical assay was used to locate tissue specific foreign gene expression in transgenic tobacco.

5.2.8.3 Toluidine blue stain. A 0.1% toluidine blue solution was used to stain unfixed, hand-cut sections of tobacco stems and leaf midribs to distinguish different cell types. Specifically, the location of phloem cells (stain pink with toluidine blue) and the xylem (stains green with toluidine blue) was sought. Tissue sections were stained with toluidine blue for 5 minutes and rinsed three times in distilled water. Results were recorded photographically.

As well as this, 0.01% toluiding blue was used to differentiate between viable and non-viable cells (P. Drennan, pers. comm.). Non-viable cells are unable to exclude the toluidine blue and stain dark purple, while viable cells exclude the dye and stain a light violet colour. The callus cells were stained, washed twice with distilled water and

viewed under the microscope. Results were recorded photographically.

5.2.8.4 Iodine stain. A Lugol's iodine solution was used to stain unfixed, hand-cut sections of tobacco stems and leaf midribs for starch deposits. The starch deposits in cells were an indication of the location of the starch sheath. After a 1 minute stain, the sections are rinsed well in distilled water. To make up Lugol's iodine, 1 g iodine and 2 g of potassium iodide are added to 300 ml of sterile water (Harrigan and McCance, 1976). Results were recorded photographically.

5.3 Results.

5.3.1 Amplification of foreign gene sequences in transgenic tobacco genomic DNA using PCR. The most important finding of the PCR analysis of genomic DNA extracted from transgenic progeny seedlings was that transgenic plants which did not express certain foreign genes nevertheless contained DNA for these genes. Also, in optimizing the PCR process, the purity of the plant genomic DNA was found to be most critical to the successful amplification of foreign sequences. Dialysis of the DNA against distilled water being an essential step in the procedure. Following the optimization of PCR conditions and plant genomic DNA purity, foreign gene sequences could be amplified in genomic DNA extracted from transgenic tobacco plants.

The results of PCR amplification of transgenic tobacco DNA with the *uidA* primers is shown in Figure 5.1. DNA extracted from kanamycin resistant progeny seedlings originating from the self pollination of parental transgenic tobacco plants 7, 11 and 3 contained *uidA* sequences which could be amplified using PCR (Fig. 5.1, lanes a, b and c). The original parental plants did not ever express the *uidA* gene, yet the *uidA* DNA is present, but also not expressed, in the progeny. When the *uidA* gene primer pair was used in a PCR reaction, the amplification products corresponded to the published size of the *uidA* amplification product (1.2 kb) based on Hamill *et al.* (1991). In Fig. 5.1, lanes j and k, which represent the kan^s progeny of plants 5 and 2, it can be seen that no *uidA* amplification product is present, as expected. These kanamycin sensitive progeny do not contain *uidA* DNA because the *nptII* and *uidA* genes co-segregate (Chapter Six).

No amount of optimization could eliminate the many amplification bands which were obtained after PCR amplification of the *sul* I gene in DNA isolated from the selfed kanamycin resistant progeny of transgenic tobacco plants 2, 4, 5, 7, 9 and 11 (Fig. 5.2). Kanamycin sensitive progeny of plants 9 and 2 were also analyzed. The gene for dihydropteroate synthase, similar to the *sul* I gene, is endogenous in plants. The endogenous dihydropteroate synthase gene probably gave rise to the many aplification products which were obtained in all the transgenic plants analyzed (Guerineau *et al.*, 1990). Following the PCR amplification with *sul* I primers, no

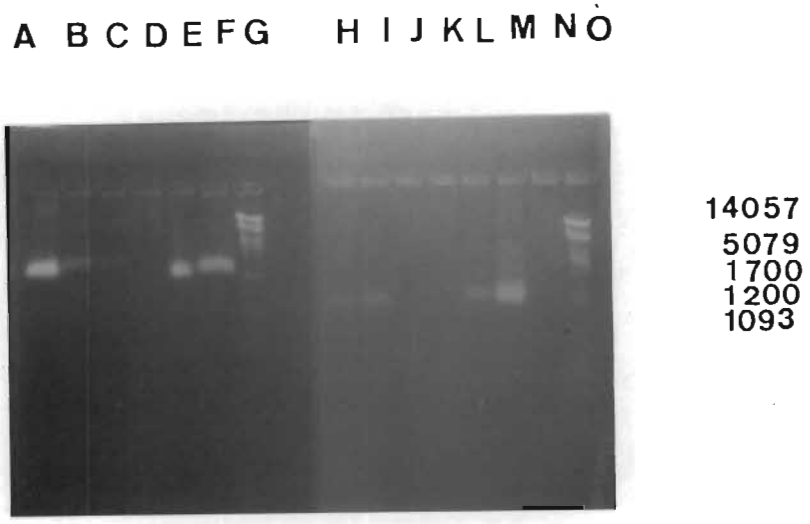


Fig. 5.1. *UidA* gene PCR amplification products. DNA extracted from the selfed progeny of transgenic tobacco plants 3, 5, 7, 9 and 11 was subjected to PCR amplification with *uidA* gene primers. The details within brackets refer to the phenotype of the transgenic progeny plants. Both kan^r and kan^s progeny of plants 2, 3, 7, 9 and 11 were analyzed using PCR. Lane a, plant 7 progeny (kan^r,GUS$^-$); lane b, plant 11 progeny (kan^r, GUS$^-$); lane c, plant 3 progeny (kan^r, GUS$^-$); lane d, no sample; lane e, plant 9 progeny (kan^r, GUS$^+$); lane f, pJIT119; lane g, lambda *pst*I molecular weight marker; lane h, plant 9 progeny (kan^r, GUS$^+$); lane i, plant 2 progeny (kan^r, GUS$^+$); lane j, plant 5 progeny (kan^s, GUS$^-$); lane k, plant 2 progeny (kan^s, GUS$^-$); lane l, plant 7 progeny (kan^r, GUS$^-$); lane m, pJIT119; lane n, no sample; lane o, lambda *pst*I molecular weight marker. Kan = kanamycin resistant phenotype, GUS$^+$ = activity of ß-glucuronidase present. GUS$^-$ = activity of ß-glucuronidase absent. r = resistant. s = sensitive. - = trait absent. + = trait present. The amplification product of the *uidA* gene is 1.2 kb.

difference could be detected between the amplification products of the *sul* I$^-$ or *sul* I$^+$ progeny of tobacco plants transformed with pJIT119, or untransformed control plants. The amplification products from the kan^r progeny of plant 3 (Fig. 5.2, lane j) had an unexplained additional high molecular weight band. None of the amplification bands obtained from the PCR of DNA from any of these plants or from the amplified pJIT119 plasmid DNA, corresponded to the expected size of 575 bp for the *sul* I amplification product (Fig. 5.2).

All the original transgenic plants contained an active *nptII* gene. The selfed progeny seedlings which survived on kanamycin medium contained the *nptII* gene. These seedlings also showed the presence of *nptII* DNA after PCR amplification (Fig. 5.3, lanes a, b, c, g, h, i, and j). The progeny which lacked the *nptII* and segregated out on medium containing kanamycin, showed that the DNA from this gene was indeed absent. No PCR amplification product was seen (Fig. 5.3, lane d).

The phenotypes of the selfed progeny from each transgenic parent plant had been previously determined by the segregation ratios and reactions of seedlings germinated on tissue culture medium containing kanamycin or asulam. This work is discussed in more detail in Chapter Six. The genotype (excluding the *sul* I gene) for each progeny class was directly determined from the PCR results. A summary of the PCR results is shown in Table 3.1.

The overall conclusion reached following the PCR analysis was that the DNA for the foreign genes *sul* I and *uidA* was present in the segregating progeny of transgenic tobacco plants, even though expression of these genes was not evident. This pattern of foreign gene inactivation was inherited by the selfed progeny from the original plants. This meant that the original transgenic plants 3, 4, 6, 7, 8 and 11 also contained the DNA for these genes even though these genes were not expressed and did not result in a transgenic phenotype. Since the DNA was present when expression was absent, DNA rearrangements, rather than gene loss, must have occurred in the original transgenic plants.

5.3.2 Preparation of sections for *in situ* hybridization. In sample preparation, the use of hand-sectioning to prepare sections of transgenic tobacco

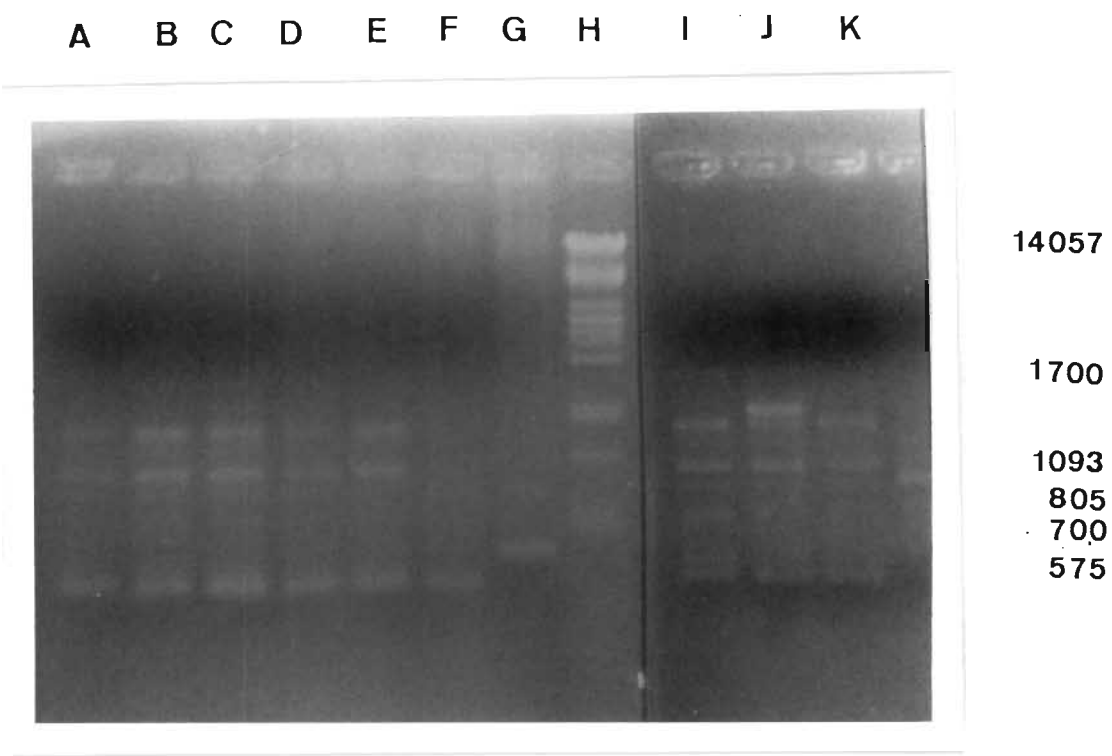


Fig. 5.2. PCR amplification of the *sul* I gene. DNA extracted from the selfed progeny of transgenic tobacco was analysed using PCR and a *sul* I primer set. The descriptions in brackets refer to the phenotype and not genotype of the plants. The original transgenic plant 9 contained two T-DNA inserts, identified through segregation analysis. Thus, kan^s progeny which were sul^r existed, as well as kan^s progeny which were sul^s. However, for this analysis, the kan^s progeny contained both sul^s and sul^r progeny. Lane a, plant 9 (kan^r sul^r); lane b, plant 2 (kan^r, sul^r); lane c, plant 5 (kan^r, sul^r; lane d, plant 9 (kan^s, sul$^{s/r}$); lane e, plant 2 (kan^s, sul^s); lane f, plant 7 (kan^r, sul^s); lane g, pJIT119; lane h, lambda *pst*I DNA molecular weight marker; lane i, plant 11 (kan^r, sul^s); lane j, plant 3 (kan^r, sul^r); lane k, plant 4 (kan^r, sul^s). Kan = kanamycin resistance. Sul = asulam resistance. s = sensitive. r = resistant. s/r = mixed sensitive and resistant progeny plants. The amplification product of the *sul* I gene is 575 bp.

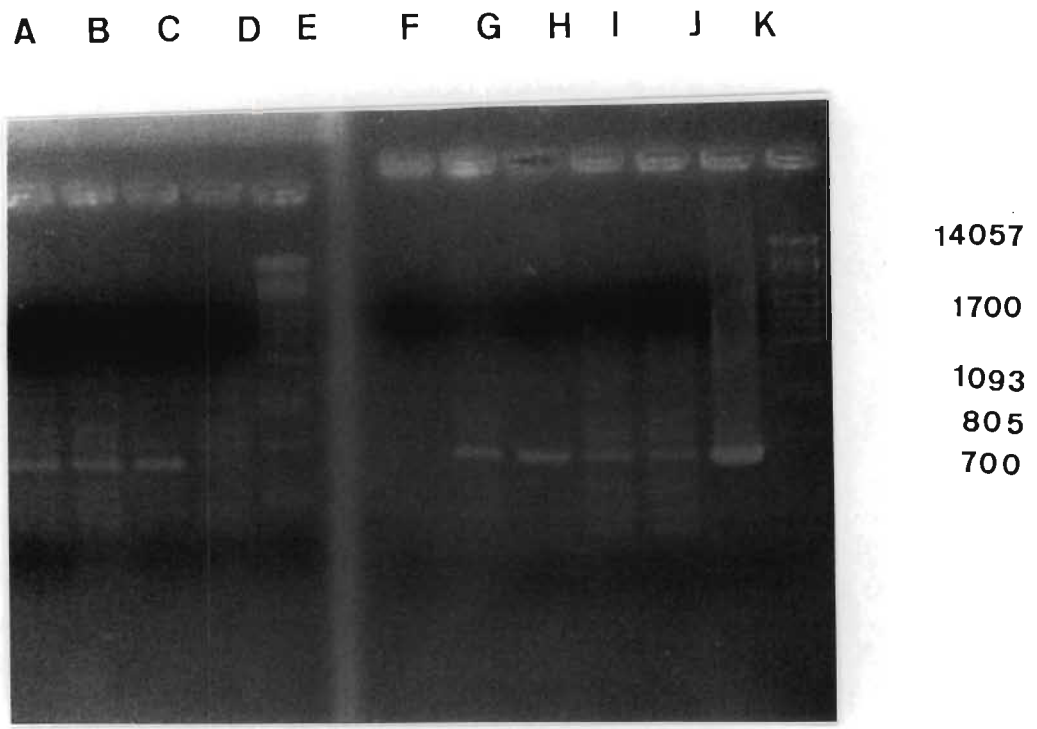


Figure 5.3. Analysis of PCR products obtained after amplification of the *nptII* gene in DNA isolated from transgenic tobacco progeny. The descriptions in brackets refer to the phenotype and not genotype of the progeny plants. Lane a, plant 9 (kan^r); lane b, plant 2 (kan^r); lane c, plant 5 (kan^r); lane d, plant 9 (kan^s); lane e, molecular weight marker; lane f, plant 2 (kan^s); lane g, plant 7 (kan^r); lane h, plant 11 (kan^r); lane i, plant 4 (kan^r), lane j, plant 3 (kan^r), lane k, pJIT119; lane l, molecular weight marker. Kan = kanamycin resistance. s = sensitive. r = resistant. The amplification product of the *nptII* gene is 700 bp.

Table 5.1. Polymerase chain reaction detection of the *npt*II and *uid*A genes in transgenic tobacco plants.

Parental plant number, and transgenic phenotype	Actual phenotype of progeny[a]	*uidA* PCR amplification product present	Inferred genotype of selfed progeny	npt II PCR amplification product present	npt II genotype of progeny
#9	kan^r GUS^+	+	$uidA^+$	+	$nptII^+$
kan^r sul^{rb} GUS^+	kan^s GUS^+	+	$uidA^+$	+	$nptII^{+c}$
	kan^s GUS^-	-	$uidA^-$	-	$nptII^-$
#2	kan^r GUS^+	+	$uidA^+$	+	$nptII^+$
kan^r sul^r GUS^+	kan^s GUS^-	-	$uidA^-$	-	$nptII^-$
#5	kan^r GUS^+	+	$uidA^+$	+	$nptII^+$
kan^r sul^r GUS^+	kan^s GUS^-	-	$uidA^-$	-	$nptII^-$
#3	kan^r GUS^-	+	$uidA^+$	+	$nptII^+$
kan^r sul^s GUS^-	kan^s GUS^-	-	$uidA^-$	-	$nptII^-$
#7	kan^r GUS^-	+	$uidA^+$	+	$nptII^+$
kan^r sul^s GUS^-	kan^s GUS^-	-	$uidA^-$	-	$nptII^-$
#11	kan^r GUS^-	+	$uidA^+$	+	$nptII^+$
kan^r sul^s GUS^-	kan^s GUS^-	-	$uidA^-$	-	$nptII^-$
TL 33	kan^s GUS^-	-	$uidA^-$	-	$nptII^-$
kan^s sul^s GUS^-					

[a] Segregation ratios were analyzed in (Chapter 6).
[b] The detection of the *sul*I gene is these plants was complicated by endogenous plant gene for dihydropteroate synthase. (Chapter 6).
[c] $^+$ = gene or trait present. $^-$ = gene or trait absent. ^{s} = sensitive. ^{r} = resistant. kan = kanamycin resistance.
sul = asulam resistance. GUS = ß-glucoronidase activity. *uid*A = glucuronidase gene. *npt*II = neomycin phosphotransferase gene

midribs gave suitably thin sections which were adequate for the *in situ* analysis of foreign gene expression. The sectioning of the apical meristem area and leaves was not as successful as that of the stems and leaf midribs. The meristems and leaves were more difficult to immobilize during sectioning.

5.3.3. Use of *in situ* hybridization to determine the presence of foreign mRNA in sections of transgenic plants which lack a transgenic phenotype. Control (non-transgenic) tissue hybridized poorly (*sul* I probe) or not at all (*uidA* probe) with the radioactively labelled probes. In control tobacco sections, small localized areas of hybridization with the[^{35}S] labelled *sul* I were probe were identified in the vascular area (Fig. 5.4). The dihydropteroate synthase enzyme is endogenous in plants, being part of the folic acid pathway. Thus, the mRNA from which this enzyme was translated was also endogenous in plants. These areas of hybridization were not significant compared to the strong hybridization of the *sul* I probe with transgenic tobacco sections (Fig. 5.7).

Probing non-transgenic tobacco sections with the *uidA* probe resulted in no non-specific areas of hybridization (Fig. 5.5). The overall colour of the *uidA* probed non-transgenic control sections was also lighter in colour than those sections made from transformed plants. No confusion could be made between non-specific hybridization and the strong hybridization pattern which resulted when transgenic tobacco sections were hybridized with the *uidA* probe (Fig. 5.8).

The [^{35}S] labelled *sul* I and *uidA* probes hybridized successfully with stem and leaf midrib sections made from individual transgenic tobacco plants. In all the transgenic plants, strong hybridization occurred with both the radioactively labelled *sul* I and *uidA* probes. Hybridization occurred in both plants which expressed the transgenic phenotype and in those which did not.

In transgenic plant 2 with the transgenic phenotype GUS$^+$sul^{r}kan^r, both the *sul* I and the *uidA* probes hybridized with the stem sections (Fig. 5.6) and with leaf midrib sections. This indicates that hybridization occurs to tobacco sections when mRNA from both the *sul* I and *uidA* genes are transcribed. When this mRNA is translated, the expected transgenic phenotype, hybridization results.

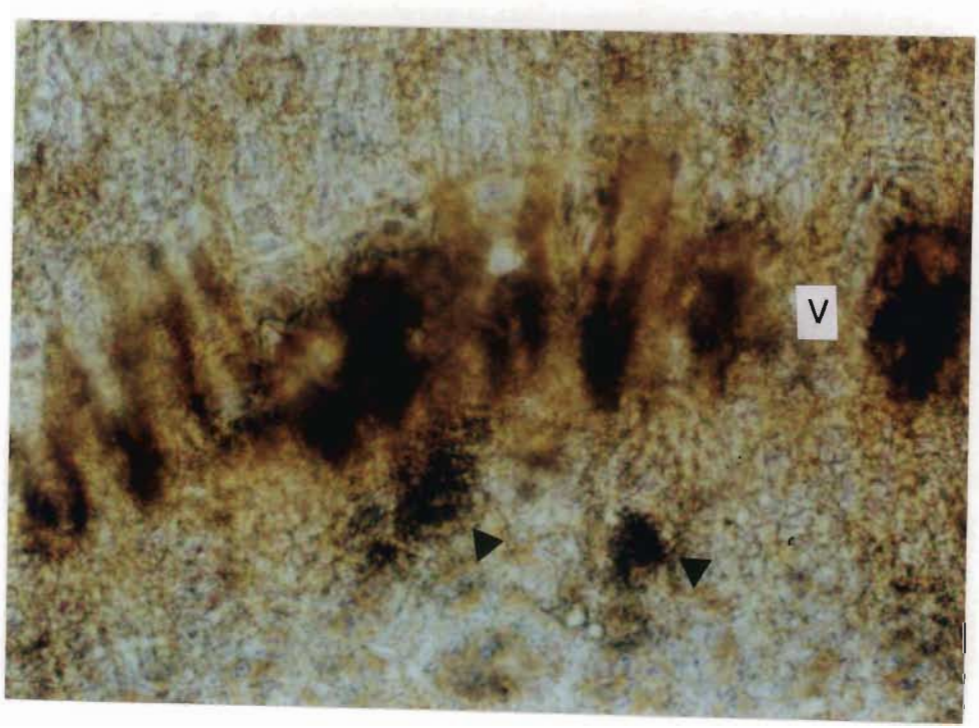


Fig. 5.4. Non-specific hybridization of the *sul* I gene probe to control untransformed tobacco sections. Localized hybridization (arrowed) corresponded to areas of endogenous dihydropteroate synthase mRNA transcription. V = vascular area of tobacco stem (x 1750).

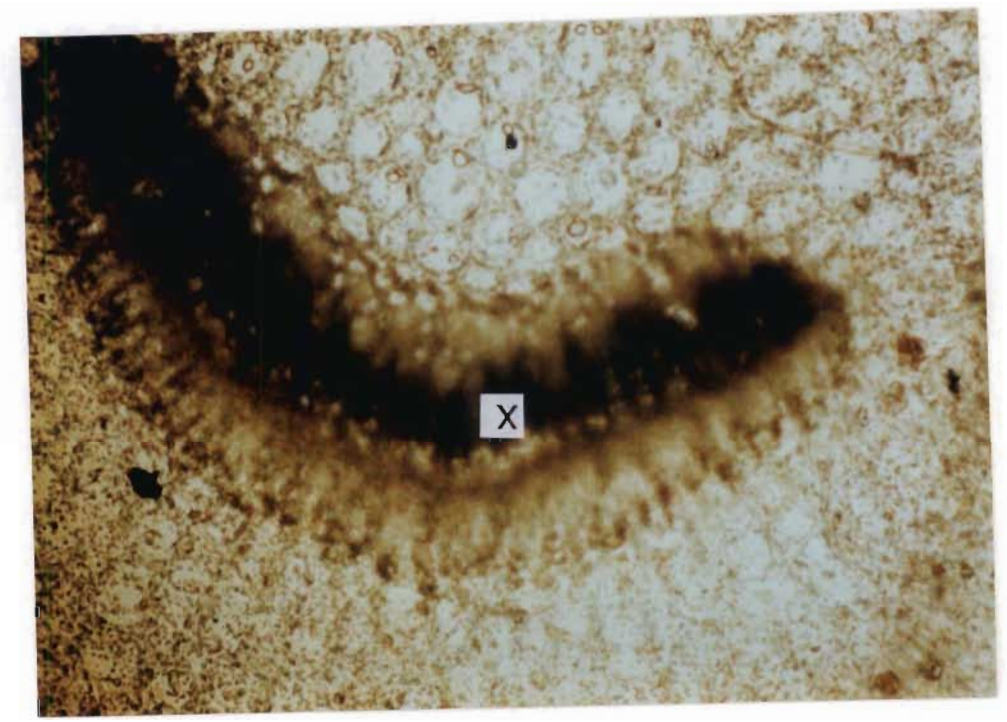


Fig. 5.5. Hybridization of the *uidA* gene probe to a control untransformed leaf midrib section. There was almost no hybridization of the probe to this section. The dark central colouration is the xylem (X) which has been sectioned obliquely. The dark granules surrounding the vascular bundle are starch grains in the starch sheath (S) (x 550).

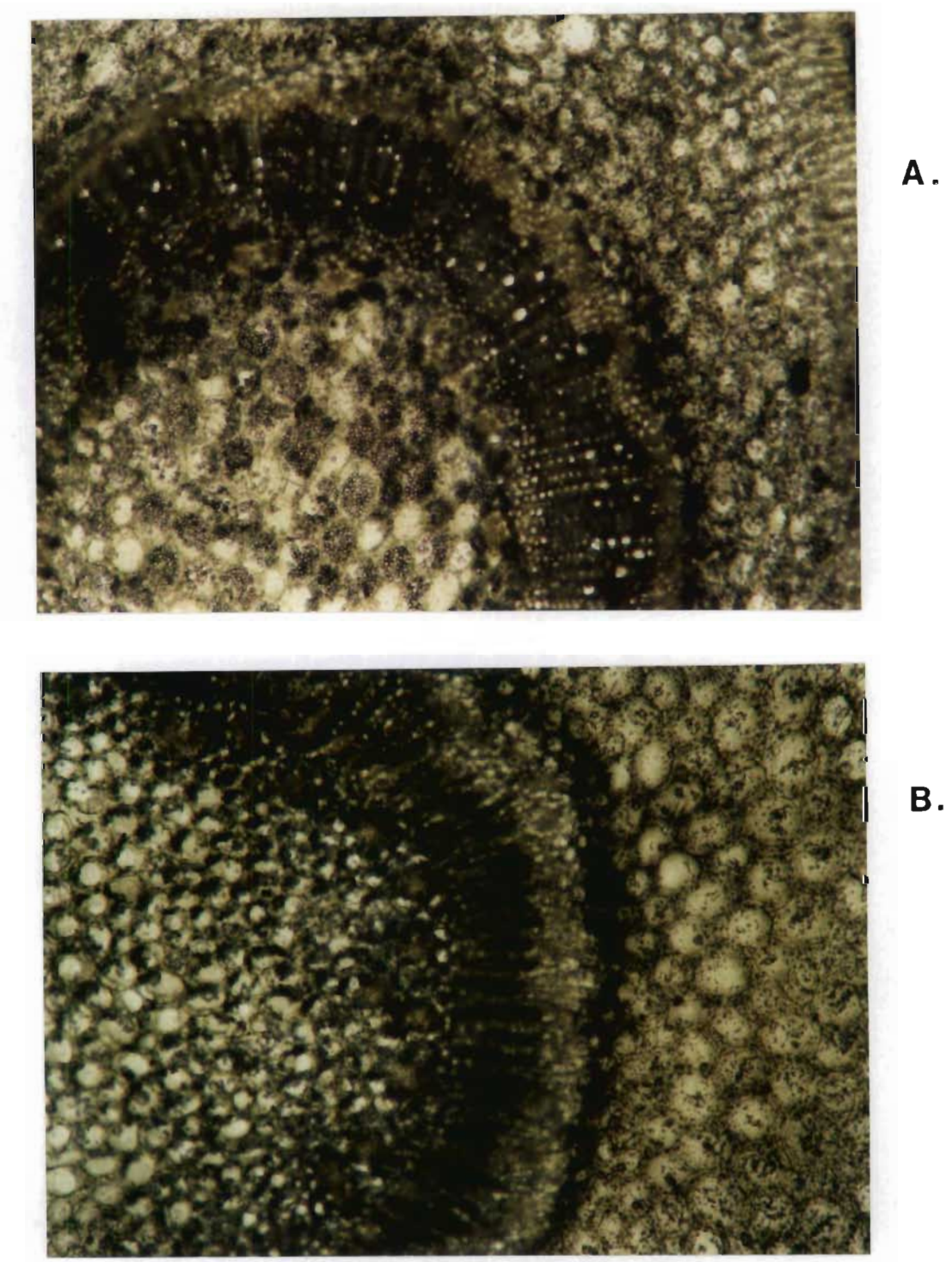


Fig. 5.6. Hybridization of two different radioactively labelled DNA probes to stem sections of plant 2. The phenotype of this plant was GUS^{+}sul^{r}kan^{r}. A; stem section probed with *sul* I probe (x 550), B: stem section probed with *uidA* probe (x 550).

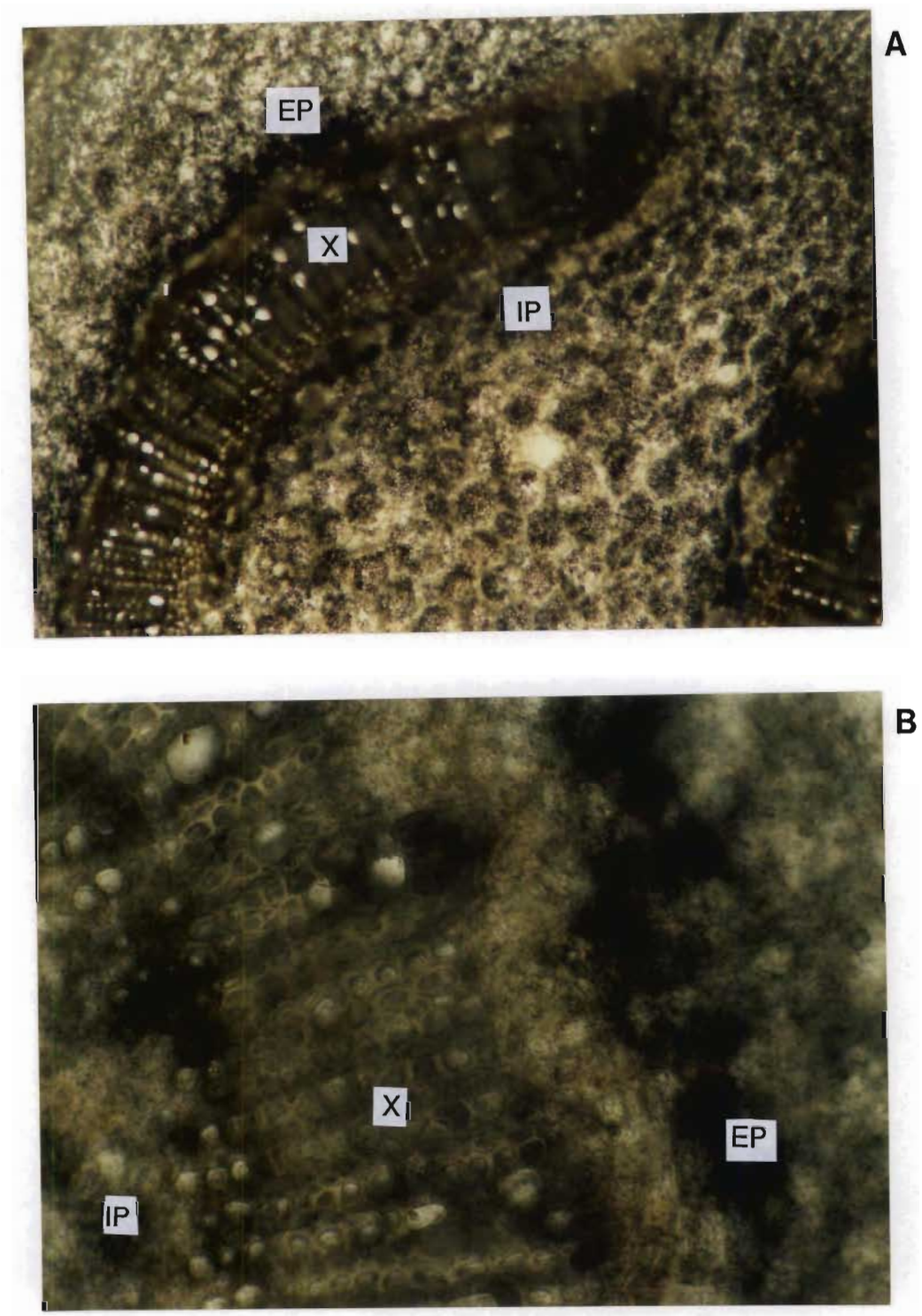


Fig. 5.7. *In situ* hybridization of radioactively labelled *sul* I gene probe to stem sections of transgenic plant 6. The phenotype of this plant was GUS$^-$*sul*^{s} kan^s. A) Stem section probed with the *sul* I probe (x 550); B) enlarged stem section showing hybridization (fine black granules) associated with the vascular area and starch sheath (x 1750). X = xylem. IP = internal phloem. E = external phloem.

expected transgenic phenotype, hybridization results.

However, in the case of transgenic plants which did not fully express the transgenic phenotype, an interesting picture emerged. For example, in transgenic plant 6 with the phenotype GUS$^-$sul^{s}kan^r, the *sul* I probe hybridized with stem (Fig. 5.7) and leaf midrib (not shown) sections. This indicated that the mRNA of the foreign gene *sul* I was transcribed in this plant even though the phenotype was absent. This finding also indicates that in this transgenic plant which does not express the asulam resistant phenotype, the gene is present and transcribed.

Another example is transgenic plant 3 which also has the phenotype GUS$^-$sul^{s}kan^r, where similar results were observed. When sections were probed with the *uidA* gene probe, the probe hybridized with stem sections (Fig. 5.8) and leaf midrib sections. This would also indicate that although there was no ß-glucuronidase enzyme produced in these plants, mRNA from the *uidA* gene was transcribed.

In the other transgenic plants (plants 5,9 and 10) which were sectioned and hybridized with the *sul* I or the *uidA*, similar results were obtained. In each plant, these two probes hybridized to sections whether or not the plants were asulam resistant or asulam sensitive, or expressed ß-glucuronidase activity or not. The pattern of hybridization was strong in each case, and not like that seen in the control, untransformed plants. Therefore, mRNA was transcribed from the *sul* I and *uidA* genes and could be detected using *in situ* hybridization, regardless of whether or not the transgenic phenotype was expressed.

5.3.4. Location of tissue specific foreign gene expression in sections of transgenic tobacco using *in situ* hybridization. In sections of transgenic tobacco plants probed with radioactively labelled DNA probes, it was very noticeable that the hybridization of radioactively labelled DNA probes occurred in highly specific areas. The vascular area of the stems (Figs. 5.7, 5.8), and midribs (Fig. 5.9) and the glandular hairs (Fig. 5.10) were clearly the main areas of hybridization. In Fig. 5.9, the predominant association of the *sul* I and *uidA* probes with the vascular bundle of a leaf

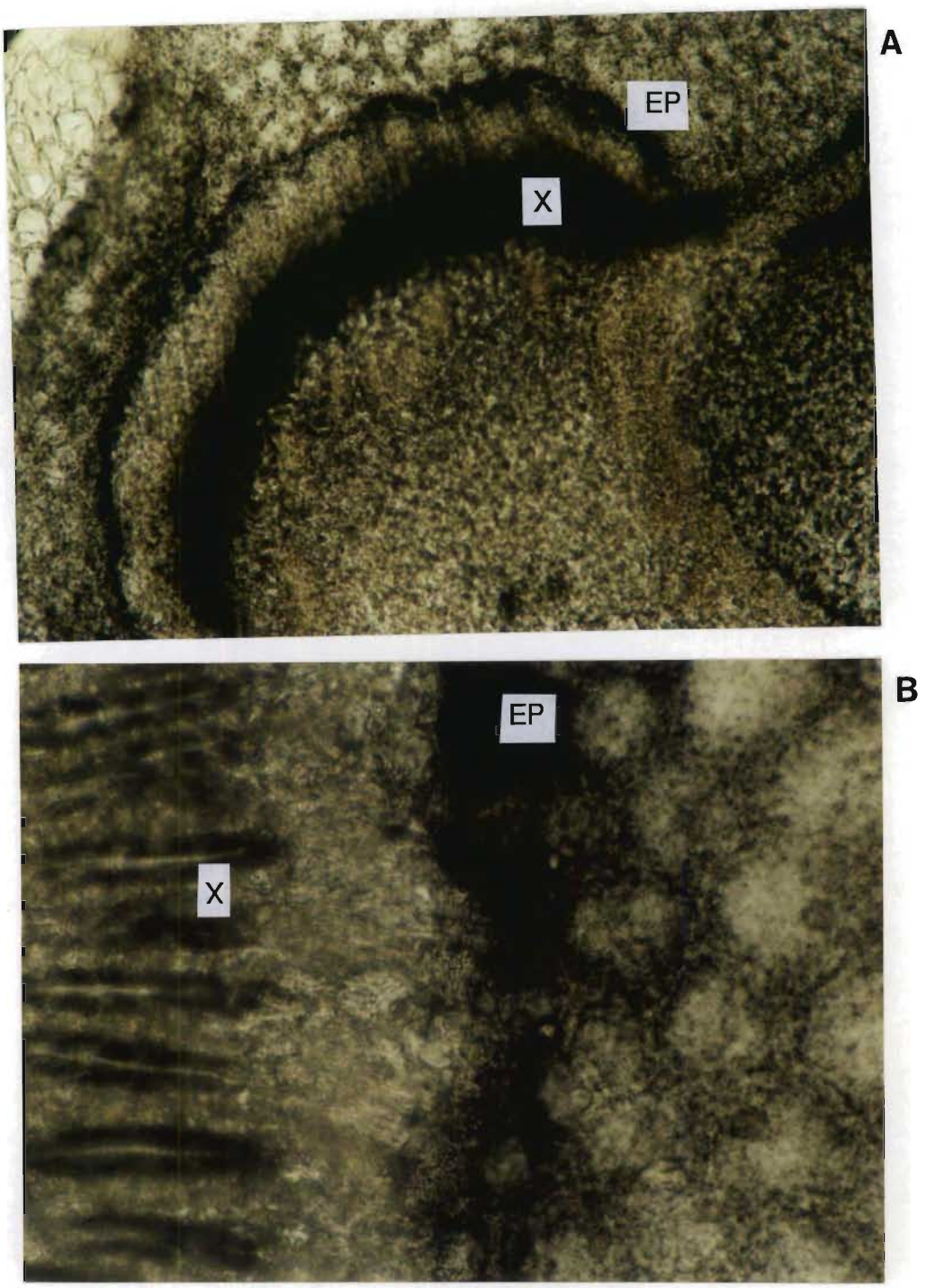


Fig. 5.8. *In situ* hybridization of a radioactively labelled *uidA* gene probe to stem sections of transgenic plant 3. The phenotype of this plant was GUS^{-}*sul*^{s}kan^{r}. A) Stem section probed with *uidA* probe showing areas of hybridization (black) (x 550); B) enlarged stem section showing hybridization (fine black granules) associated with the vascular area (x 1750). X = xylem. EP = external phloem.

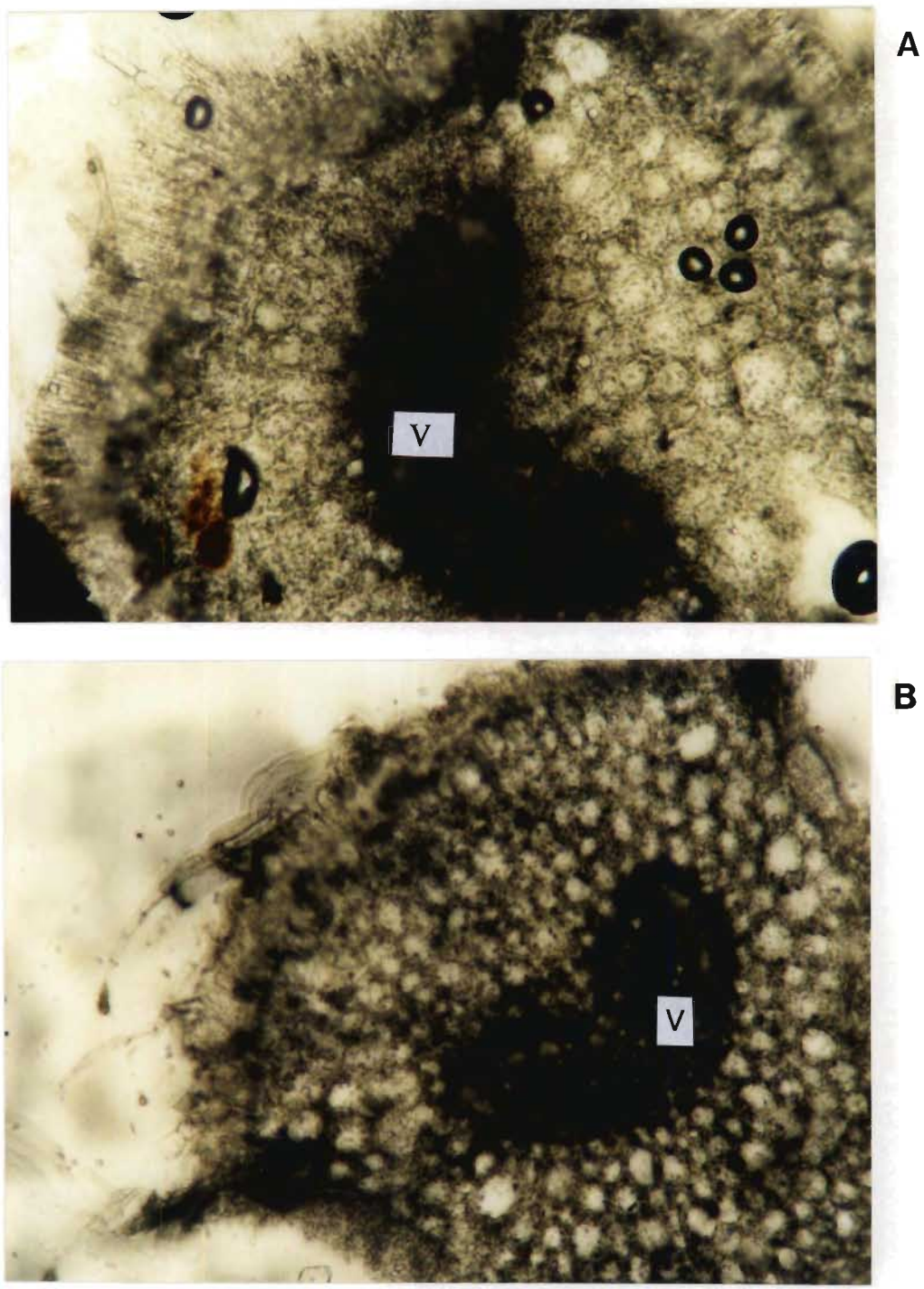


Fig. 5.9 *In situ* hybridization of radioactively labelled DNA probes to leaf midrib sections of transgenic plant 9. A) Midrib section probed with the *uid*A probe; B) midrib section probed with *sul* I probe (X550). The probes hybridized strongly with the crescent shaped vascular bundle of the midrib. V = vascular bundle.

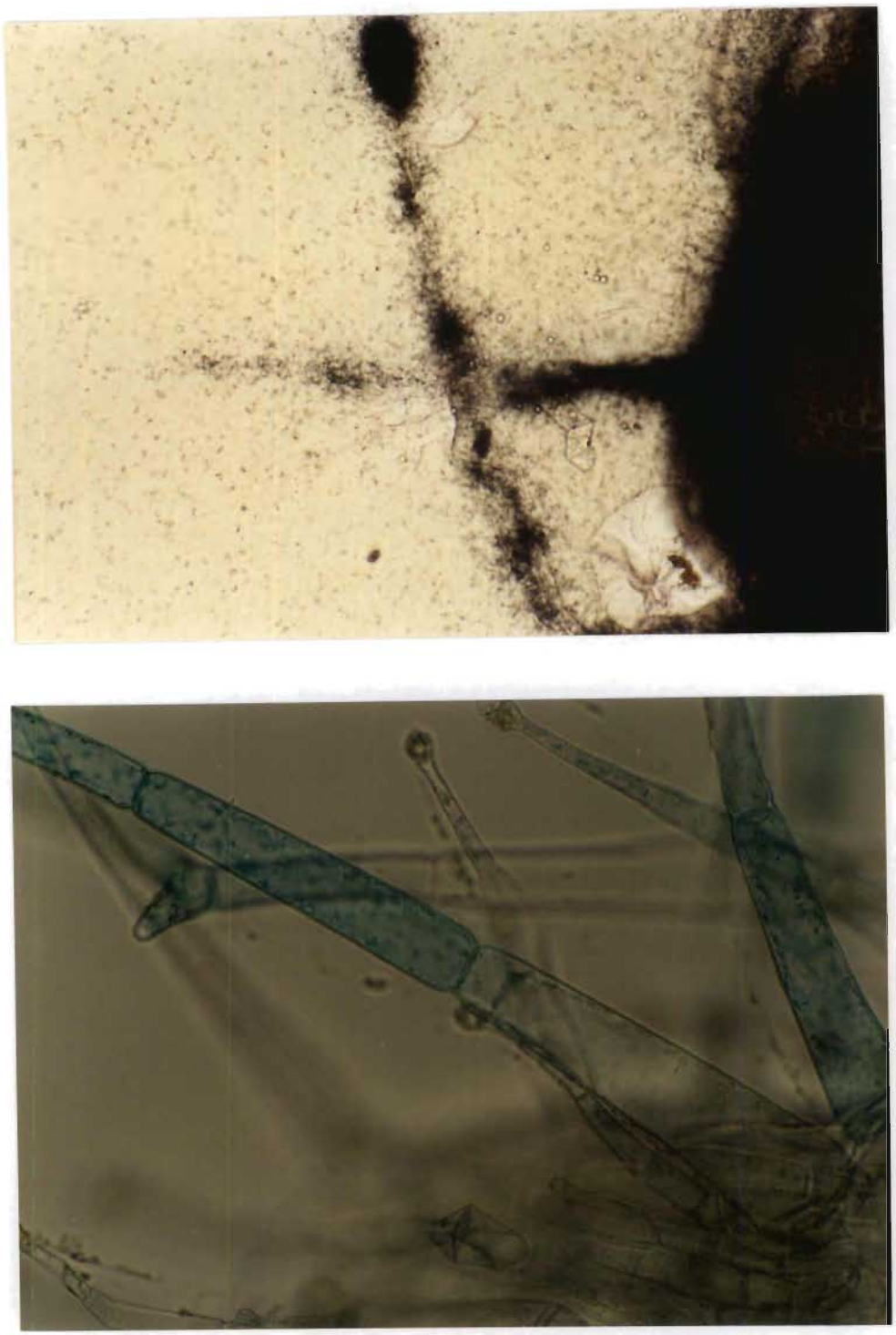


Fig. 5.10 Example of foreign gene expression in transgenic tobacco glandular hairs. A) Example of a typical hybridization pattern of a radioactively labelled gene probe to glandular hairs; B) glandular hairs assayed with the GUS histochemical assay (x 550).

midrib section can be clearly seen. The same pattern of association of the *uidA* and *sul* I probes with the vascular areas of transgenic tobacco stem sections (Figs. 5.6, 5.7 and 5.8). The glandular hairs of all transgenic tobacco plants showed strong hybridization when probed with the *sul* I or *uidA* probes (Fig. 5.10).

The hybridization was very dark in all these areas. Although hybridization was observed in the cortex or pith areas of stems and midribs, it was never as intense as the deposition around the vascular area. Sections of apical meristems of the individual transgenic plants also showed that hybridization was associated with the vascular tissue in every case (data not shown).

When the tobacco sections were viewed under a high magnification, heavy deposits of silver grains could be seen to be finely distributed over both the internal and external phloem cells (Figs. 5.7 and 5.8). Because the hybridization pattern outside the external phloem appeared to consist of a single dark line (Figs. 5.6, 5.7 and 5.8), the hybridized stem sections were scrutinized very carefully to determine whether the hybridization to the exterior of the xylem was associated with the external phloem and/or the starch sheath. The starch sheath cells form a continuous line around the external phloem (Fig. 5.11). Also, stem sections treated with the GUS histochemical assay (Fig. 5.12), stems sections stained with toluidine blue (Fig. 5.13), and stem section stained with iodine (Fig. 5.11) were carefully examined to identify the cells that corresponded to those which hybridized strongly in the *in situ* hybridization experiment.

The GUS histochemical assay of stem sections showed, however, that GUS activity was only associated with the internal and external phloem, and not with the starch sheath. In some older stem sections, the external phloem appears as a continuous cell layer (Fig. 5.14), while in others which may be younger, it appears as groups of cells, as shown in Fig. 5.12. The *in situ* hybridization was carried out on older stem sections, and this may explain why the area of hybridization external to the xylem appears as a continuous line. The radioactive probes did not hybridize to the starch sheath in these sections, but to the external phloem. Also, in sections of tobacco stem which were stained with the GUS histochemical assay and then with Lugoll's iodine the

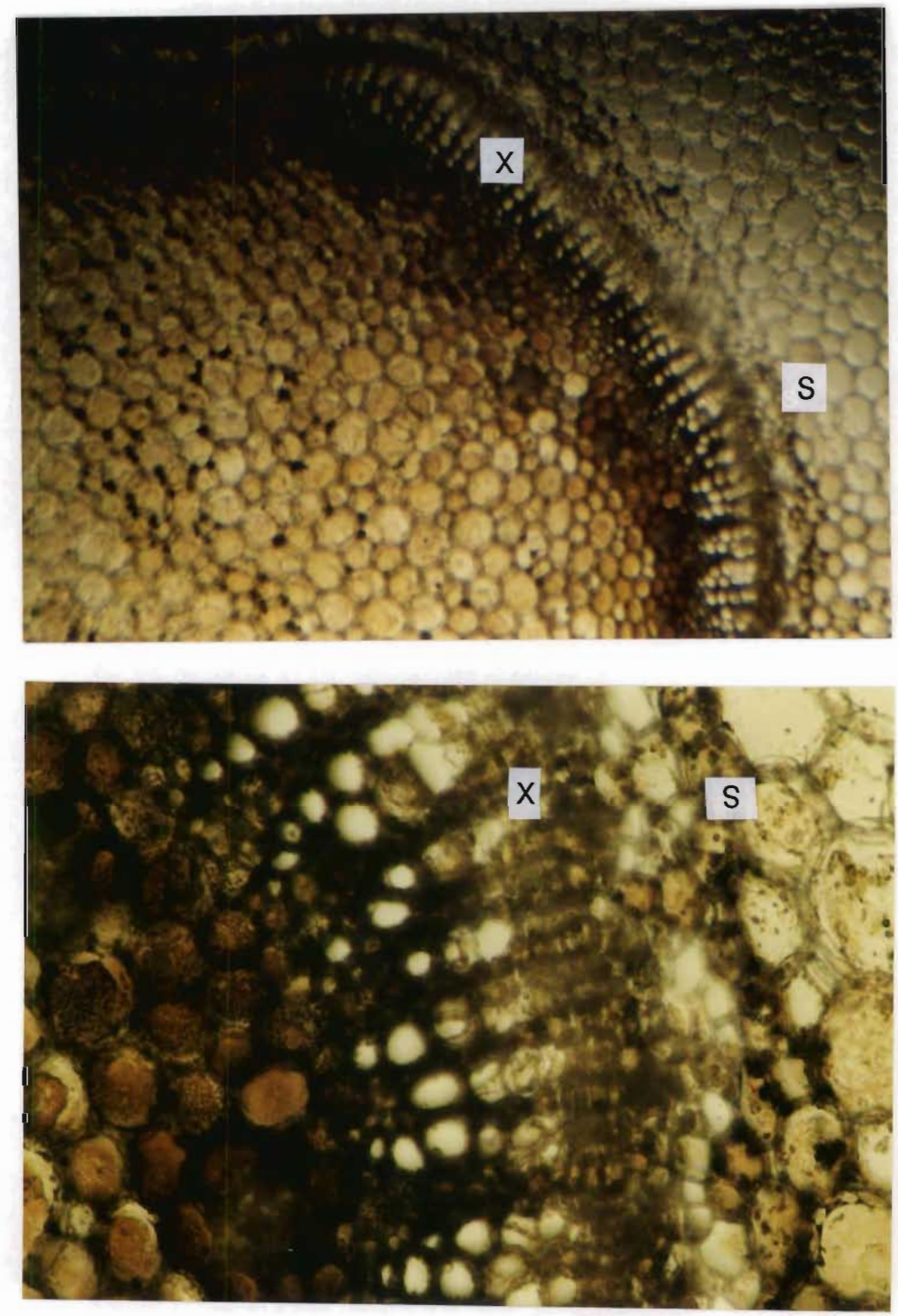


Fig. 5.11. Tobacco stem sections stained with iodine. The iodine stain distinguished the starch layer from the surrounding tissues by staining the starch grains dark brown. A; Stem section (x 550), B; enlarged stem section with starch sheath clearly distinguished by the presence of numerous starch grains within each cell (x 1750). S = starch sheath. X = xylem.

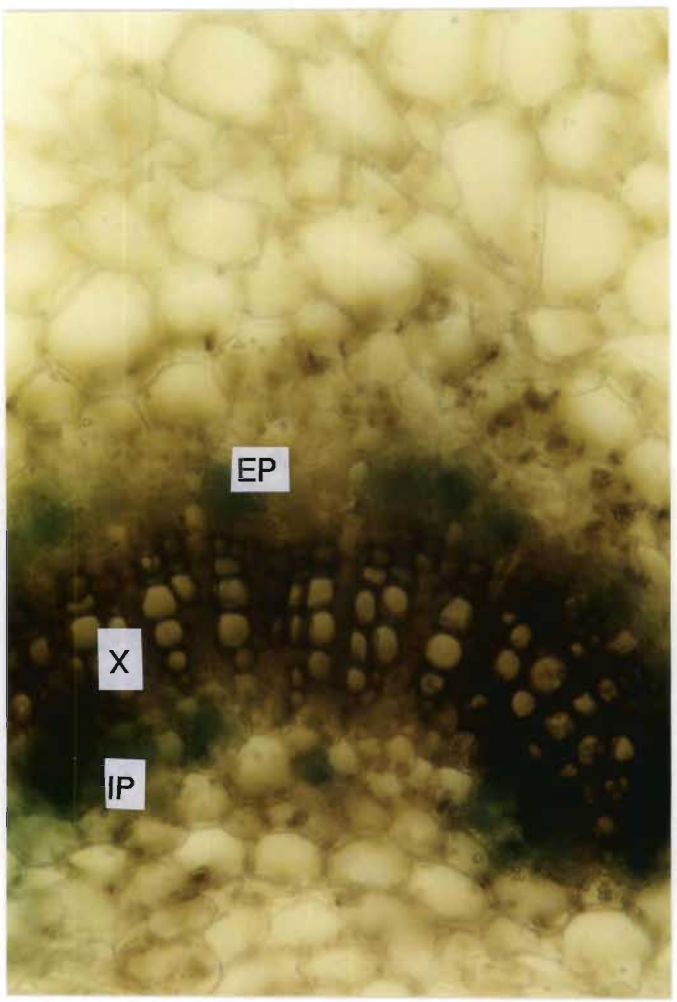


Fig. 5.12. GUS histochemical assay of transgenic tissues of plant 9. Stem section after GUS histochemical assay. GUS activity (blue colour) is clearly associated with the vascular area, especially with the internal phloem (IP) and external phloem (EP). X = xylem. (x 550).

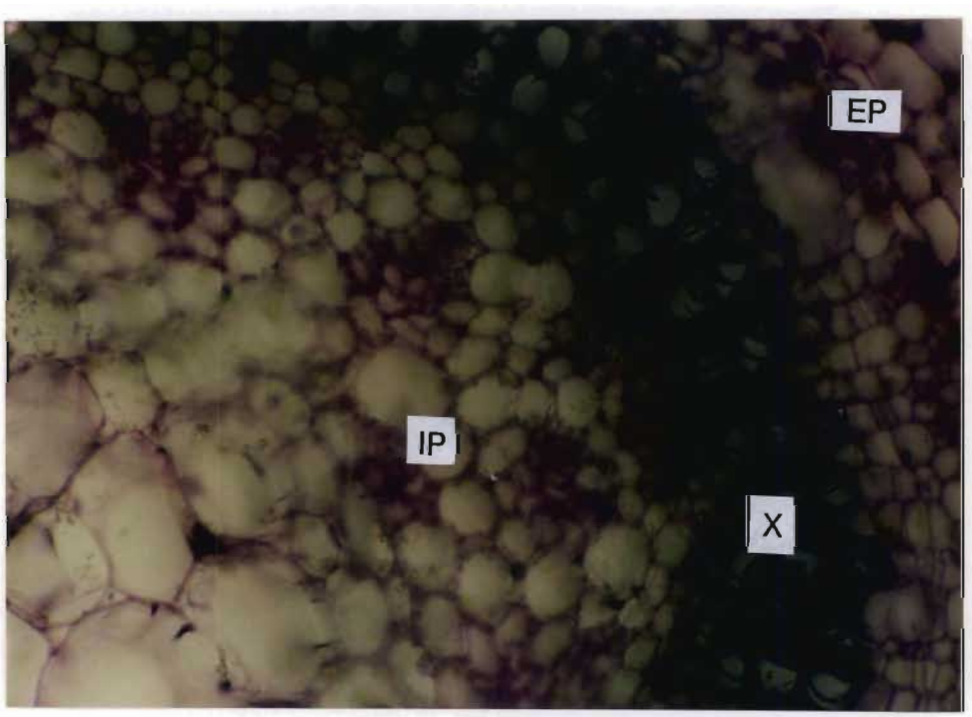


Fig. 5.13.. Tobacco stem section stained with toluidine blue. Toluidine blue stains living cells a pink colour and non-living cells (e.g. xylem) a blue colour (x 875). IP = internal phloem. EP = external phloem. X = xylem.

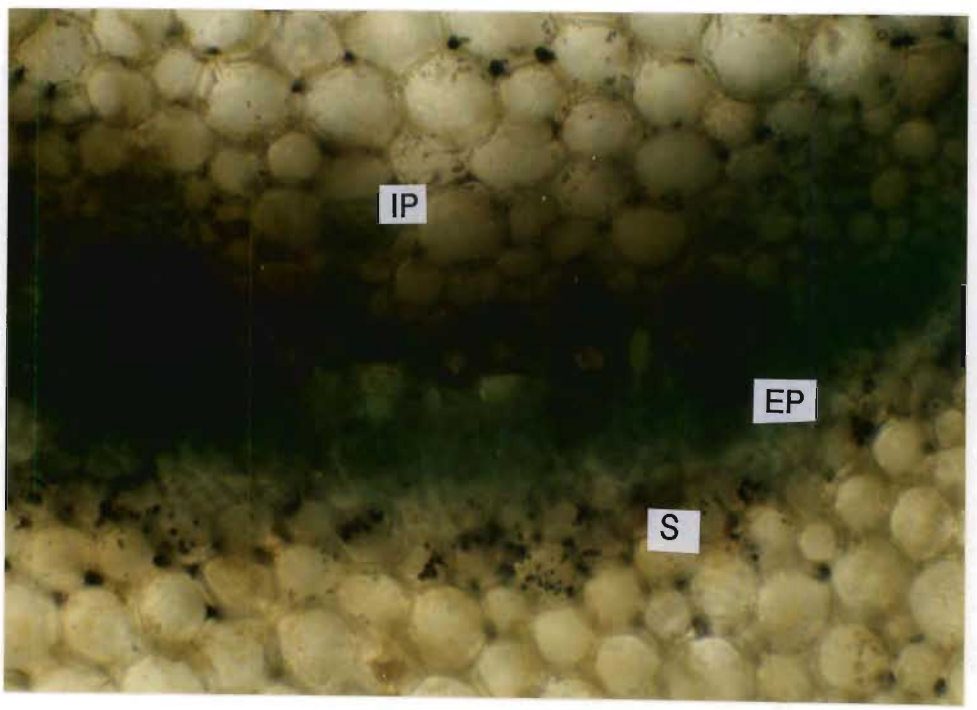


Fig. 5.14. Transgenic tobacco stem section showing location of GUS activity and the starch sheath, following the GUS histochemical assay and iodine staining. The internal (IP) and external (EP) phloem stain blue with GUS activity, while the starch sheath (S) containing black starch grains does not stain blue (x 440).

GUS negative starch sheath can clearly be seen lying just outside the GUS positive phloem band (Fig. 5.14). The *uidA* gene under 35S promoter control was not active in the starch sheath, but only in the phloem (external and internal) and glandular hairs. GUS activity under 35S promoter control was not seen in any other structures or tissues of normal, unstressed mature tobacco plants.

In stem and midrib sections of the remaining transgenic tobacco plants 2, 3, 5, 6, 9 and 10 , the hybridization of radioactively labelled DNA probes was also found strongly associated with the vascular area in each case (data not shown). The pattern of hybridization was therefore constant in all the transgenic tobacco plants analyzed.

5.3.5 Location of foreign gene (*uidA*) expression in sections of transgenic tobacco plants using the GUS histochemical method and biological stains.

5.3.5.1 GUS histochemical assay. A GUS histochemical assay of transgenic tobacco stem sections showed that the localization of GUS activity was around the vascular bundle, specifically associated with the internal and external phloem cells (Fig. 5.12). Interestingly, no GUS activity is seen associated with the starch sheath, in this figure. This finding contrasted with the *in situ* hybridization results, and hence additional sections were made and stained for GUS activity, and using toluidine blue and iodine to clarify the location of GUS gene expression.

GUS activity was seen in the glandular hairs (Fig. 5.10). No GUS activity was seen in the cortex, pith, or epidermis of any of the hardened off transgenic plants assayed (Fig. 5.15).

5.3.5.2 Toluidine blue stain. The results of toluidine blue and iodine staining was used to define the tissues surrounding the vascular bundle in the tobacco stem, where intense hybridization was observed at such high intensity. The toluidine blue stain revealed clearly the xylem cells (blue) and the phloem cells (pink) to the interior and exterior of the xylem within the vascular bundle (Fig. 5.13).

5.3.5.3 Iodine staining. The iodine stain identifying the cell layer external to the

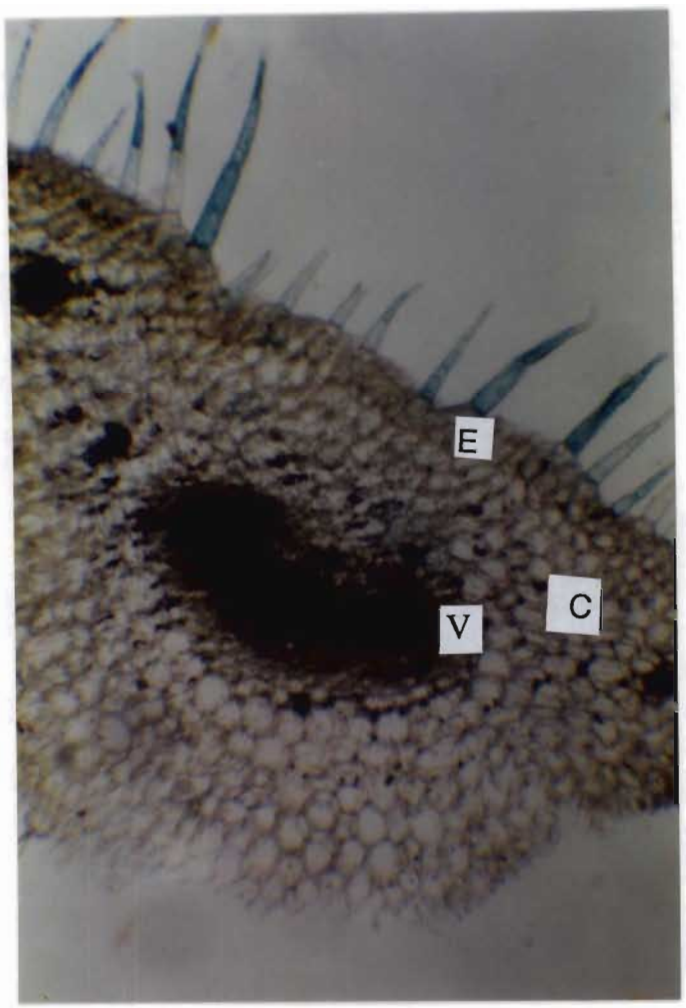


Fig. 5.15 Section of a mature transgenic tobacco midrib showing absence of GUS activity in cortex (C) and epidermis (E). The GUS activity is located around the vascular bundle (V) of the stem section (X550).

phloem as the starch sheath through staining the starch grains within the cells (Fig. 5.11). The iodine stain was applied to sections of tobacco stem already stained for GUS activity to clearly demonstrate the separateness of the external phloem and starch sheath (Fig. 5.14).

5.3.5.4 Non-tissue specific foreign gene *uid*A expression in transgenic tobacco callus. Transgenic tobacco callus cells were strongly GUS positive (Fig. 5.16 (A)) although they are dedifferentiated cells. In contrast, in whole transgenic tobacco plants transformed with a *uidA* gene under 35S promoter control, only the phloem cells and glandular hairs expressed the *uidA* gene under the control of the CaMV 35S promoter. In the callus samples which were assayed for *uidA* activity, there was a noticeable variation in GUS expression, with not all of the cells in the callus culture expressing the GUS gene to the same extent (Fig. 5.16 B). This difference in expression levels could have related to the viability of the cells, with non-viable cells failing to express GUS. The toluidine blue stain was used to determine the proportion of callus cells which were non-viable. Living and non-living tobacco callus cells could be clearly distinguished by toluidine blue staining (Fig. 5.16 C). Many samples of toluidine blue stained cells were examined and the callus cells in each case were almost entirely viable (Fig. 5.16 D). This indicated that in a sample of cells assayed for GUS expression, the major proportion of cells are also viable. The variation in the GUS expression in callus cell samples seen was, therefore, not due to the cells being non-viable, but to other factors.

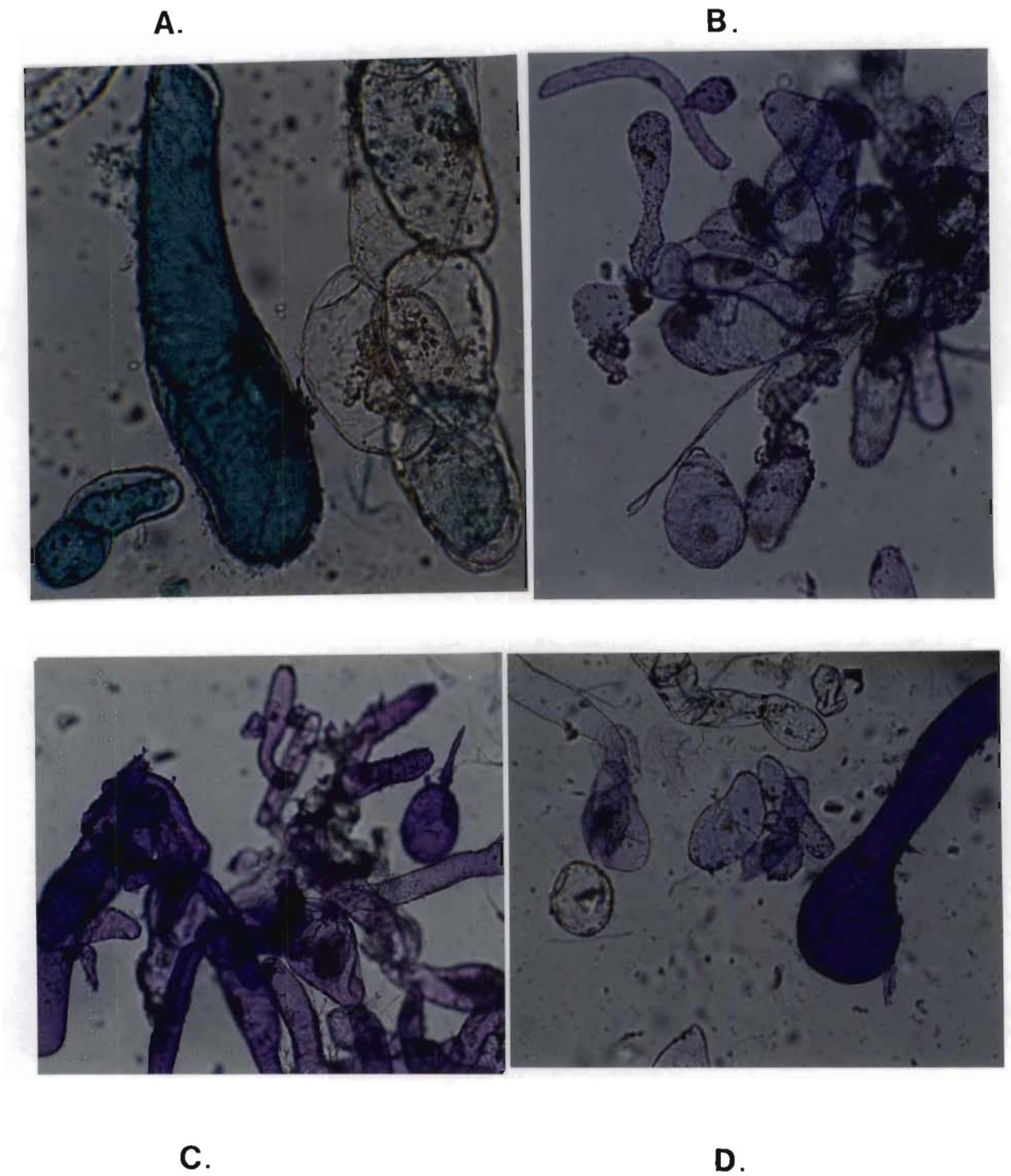


Fig. 5.16 Study of foreign gene (*uidA*) expression in tobacco callus cells. A) GUS positive transgenic tobacco callus cells (x 1750); B) toluidine blue exclusion by viable cells. Living tobacco callus cells stain violet (x 1750); C) toluidine blue uptake by heat killed tobacco callus cells. Dead cells stain dark purple (X 1750); D) Mixed living and heat killed cells, showing the difference in toluidine blue staining (x 1750).

5.4 Discussion

When transgenic plants are produced following *Agrobacterium*-mediated leaf disc transformation, single cell or protoplast transformation, the individual plants can vary extensively in their phenotype, even when leaf material from the same individual plant is used to provide explant material for transformation. Each transgenic plant obtained from *Agrobacterium*-mediated leaf disc transformation, single cell or protoplast transformation, arises from a separate transformation event (Draper *et al.*, 1988). Also, as the T-DNA insertion is random, a different genomic location of each T-DNA insertion is possible (Offringa *et al.*, 1990). This, and the various modifications of the T-DNA which can occur during or after transformation, cause inter-transformant variability (Breyne *et al.*, 1992).

In this study, ten transgenic tobacco plants produced from an *Agrobacterium*-mediated leaf disc experiment were studied with regard to foreign gene presence and expression. The high rate of foreign gene inactivation in the transgenic tobacco plants suggests that this is a common occurrence in the production of transgenic plants. The reasons for the inactivation of foreign genes in these plants were investigated in this study. Foreign genes can be inactivated in transgenic plants through foreign DNA rearrangements, foreign DNA loss, the "position effect" and DNA methylation.

In each transgenic tobacco plant which lacked the expression of one or more of the foreign genes, i.e. lacked the expected transgenic phenotype, foreign gene rearrangements, rather than foreign gene loss was deduced through the use of the polymerase chain reaction and from *in situ* hybridization studies.

The polymerase chain reaction showed that within the genomic DNA extracted from individual transgenic plants (with or without the expected foreign phenotype), foreign genes were present and could be detected. This foreign DNA corresponded to the expected amplification product size. This finding indicated that the DNA from each foreign gene was present, and that no major deletions or duplications had occurred within the amplified sections. Rearrangements outside these regions would be undetected using PCR.

In situ hybridization to locate foreign mRNA within sections of transgenic tobacco plants without the expected transgenic phenotype, showed that in each transgenic tobacco plant studied, foreign gene transcription occurred. Specific mRNA from each type of foreign gene could be located within the transgenic plants, whether or not the transgenic phenotype was present.

The presence of foreign mRNA in the transgenic plants without the full transgenic phenotype, discounted the "position effect" as a reason for foreign gene inactivation in these plants. No mRNA is produced when the genomic site of T-DNA integration prevents transcription. The *in situ* hybridization results indicated that transcription of the foreign genes *uidA* and *sul* I did occur, and foreign mRNA was present, even in transgenic plants where the transgenic phenotype was absent. The "position effect" would also be expected to result in inactivation of an entire T-DNA insert, and apply to all the genes encoded by the T-DNA. The "position effect" would not cause the selective inactivation of certain genes on the T-DNA as observed in this study.

Methylation of foreign genes was also discounted as a reason for foreign gene inactivation observed in this study. Methylation can selectively inhibit the expression of foreign genes within a T-DNA insert, but during this inhibition, no foreign mRNA will be produced. Again, foreign mRNA was produced in transgenic tobacco plants lacking a transgenic phenotype investigated in this study, as indicated by the *in situ* hybridization results.

In this study, each of the ten transgenic plants studied had undergone foreign gene inactivation, possibly through DNA rearrangements. Each of the ten transgenic plants which had an active *nptII* gene, had either an inactive *sul* I or *uidA* gene. The level of "escape" plants (caused by *nptII* gene inactivation) which were originally discarded was unacceptibly high (approaching half the original plantlets obtained in this study). The high percentage of foreign gene rearrangements affecting the expression of foreign genes in transgenic plants is a considerable inconvenience. Transgenic plants obtained from a transformation experiment require screening in some detail to determine whether they are fully transgenic, and whether they express those foreign genes not selected for using selectable markers (usually *nptII*) during the initial

selection procedures.

Possibly in the future, as the mechanism of gene alteration during *Agrobacterium*-mediated transformation is fully elucidated, some steps will be made to design vector plasmids with T-DNA and genes which resist these deleterious rearrangements. Perhaps at that stage, all of the transgenic plants obtained in a transformation experiment will be guaranteed to express all the foreign genes.

In the future, it is also likely that a more refined use of gene targeting strategies will allow a more controlled approach to plant genetic engineering. Through gene targeting, it may soon be possible to control the exact location of T-DNA insertion, eliminating "position" effects (Offringa *et al.*, 1992). The T-DNA could be targeted to an area of the genome where its expression is guaranteed (Offringa *et al.*, 1992).

A central problem in research on foreign gene regulation in transgenic plants is the mechanisms of tissue- or cell-type-specific gene expression. The cauliflower mosaic virus (CaMV) 35S promoter is currently the most widely used general promoter for gene expression in transgenic plants. The CaMV 35S promoter has been shown to be highly active in most plant organs and during most stages of development when integrated into the genome of transgenic plants (Benfey *et al.*, 1989). It was initially regarded as a constitutive promoter (Jefferson *et al.*, 1987), but now this description must be qualified. The 35S promoter is now known to possess a highly tissue specific location of activity (Jefferson *et al.*, 1987; Benfey *et al.*, 1989; Benfey *et al.*, 1990).

Investigations of the 35S promoter of the cauliflower mosaic virus (CaMV) demonstrated that this promoter is modular and is comprised of several *cis* elements, each of which confers cell-type-specific gene expression in plants (Benfey *et al.*, 1989; Benfey *et al.*, 1990). This modular assembly of *cis* element probably applies to other plant promoters as well.

In this study, the probing of transgenic tissue sections with radioactive DNA probes using *in situ* hybridization, as well as carrying out the GUS histochemical assay on tobacco sections, clearly showed the strongly tissue specific nature of foreign gene

expression under the control of the CaMV 35S promoter. Foreign gene activity, detected either through deposits of silver grains located where a radioactively labelled probe had detected foreign mRNA, or through GUS activity, could be located predominantly in the phloem cells and glandular hairs of transgenic tobacco. The specificity of foreign gene activity under CaMV 35S promoter control corresponded to that reported in the literature i.e. generally associated with the vascular tissues of the various transgenic plant organs (Benfey *et al.*, 1989; Terada and Shimamota, 1990; Omirulleh *et al.*, 1992; Omirulleh *et al.*, 1993). It was thought that the pictorial evidence of the tissue specificity of foreign gene activity under the control of the 35S promoter obtained in this study was novel. Most of the studies showing tissue specificity of foreign gene expression use the GUS histochemical assay to localize foreign gene expression (Köster-Töpfer *et al.*, 1989; Terada and Shimamoto, 1990).

During the analysis of the results of the *in situ* hybridization experiments, it was thought that hybridization also occurred in the starch sheath of the tobacco plants, however, through studies with the GUS histochemical assay and iodine staining, it was found that activity was restricted to the external phloem, which lies immediately below the starch sheath and to the internal phloem and glandular hairs. The observed tissue specific effect was not restricted to one transgenic plant, but was observed in all ten transgenic plants studied.

The use of *in situ* hybridization to demonstrate the tissue specificity of foreign gene expression under CaMV 35S promoter control is novel. Usually, ß-glucuronidase activity is used to locate foreign gene expression within transgenic tissues (Benfey *et al.*, 1990; Terada and Shimamoto, 1990; Weber *et al.*, 1990; Bochardt *et al.*, 1992; Ottoviani *et al.*, 1993; Omirulleh *et al.*, 1993). The GUS histochemical assay, although yielding information rapidly about the location of foreign gene (*uidA*) expression, nevertheless only reports on the activity of one gene, the *uidA* gene. *In situ* hybridization can give reliable information about any gene, foreign or endogenous, to which a DNA or RNA probe can be made.

The interpretation of the GUS histochemical staining is sometimes not simple

(Jefferson *et al.*, 1987; Terada and Shimamoto, 1990). Closely packed small cells such as phloem cells tend to stain a more intense blue because the number of cells per unit area is greater (Jefferson *et al.*, 1987). Therefore, the distinctive staining pattern observed in various tissues of transformed plants may not necessarily reflect differences in the level of expression (Jefferson *et al.*, 1987; Terado and Shimamoto, 1990). In this study, the size of cells expressing GUS activity under 35S promoter control did not affect the intensity of blue obtained, as stated by Jefferson *et al.* (1987). Both large and small cells (callus samples) could contain faint or intense blue colouration. Other factors affecting the amount of ß-glucuronidase production must be at work. Also, the tissue specificity of *uidA* gene expression under CaMV 35S promoter control is dependent on factors other than the position of the cells within the stem or leaf midrib.

The CaMV 35S promoter is preferentially active in cells during the S-phase of the cell cycle (Nagata *et al.*, 1987). This may mean that expression of CaMV-GUS may simply reflect active cell division. Also, different cell-types within plants are expected to have differing metabolic activity with corresponding differences in rates of transcription and translation (Jefferson *et al.*, 1987), and so the tissue specificity of CaMV-GUS may reflect these differences.

The use of *in situ* hybridization to detect the location of foreign gene expression can give more reliable results. The mRNA is cross-linked to proteins to prevent its displacement during processing and this step could present a more accurate location of foreign gene. In thin sections, the blue pigment of the GUS histochemical assay may wash away during the assay, resulting in less GUS activity than expected.

Although the activity and structure of the CaMV 35S promoter has been extensively reported on, an aspect of this promoter is not often emphasized. The properties of the promoter reflect the nature of the CaMV virus from which it is derived. The tissue specific location of activity of the CaMV 35S promoter must have an adaptive value to the virus, and play a role in viral replication. In infected plants, the 35S RNA is the major CaMV RNA species. This RNA covers the complete viral genome, encoding all the proteins produced by the virus, and also functions as a template for reverse

transcription during the viral replication cycle (Fütter *et al.*, 1990). The vascular tissue of host plants is a key site of interaction between CaMV and its host for dissemination of virus particles through the plant (Covey *et al.*, 1990). Also, CaMV is aphid transmitted in nature. Aphids are phloem feeders, inserting their stylets into phloem cells to obtain nutrients. Also, other plant DNA viruses, the geminiviruses, also replicates in the phloem parenchyma of host plants (Jefferson *et al.*, 1987). The replication of the virus (and hence the expression of the CaMV 35S promoter) in rapidly metabolizing and dividing phloem cells would ensure the survival of the virus. The CaMV 35S promoter reflects this survival strategy, even though removed from the virus.

In plant genetic engineering, a promoter with a strong tissue specifity of action may not always be desirable. Although the CaMV 35S promoter is commonly used in plant genetic engineering, the confinement of its activity to vascular tissue could be a disadvantage. Unless a systemically acting agent is to be counteracted by foreign genes e.g. herbicides or plant viruses, vascular tissue-specific foreign gene activity may not be effective. Where the agent to be counteracted by foreign genes is non-systemic i.e. a insect pest or fungal pathogen, a wound-inducible promoter might be more beneficial. Also, the constitutive nature of the 35S promoter may be wasteful. Non-constitutive promoters such as the heat shock, wound-inducible or various seed-specific promoters would enable foreign gene activity to be activated when and where needed, rather than allowing wasteful continual foreign gene expression.

Chapter 6

The expression of foreign genes in the reproductive structures (*uid*A) and progeny (*uid*A, *sul* I and *nptII*) of transgenic tobacco plants, as well as stress and developmental regulation of *uidA* gene expression under CaMV 35S promoter control.

Chapter 6

The expression of foreign genes in the reproductive structures and progeny (*uid*A, *sul* I and *nptII*) of transgenic tobacco plants, as well as stress and developmental regulation of *uidA* gene expression under CaMV 35S promoter control.

6.0 Summary

Of critical importance in the development of transgenic plant lines for a plant breeding programme is that the plants are fertile and transmit actively expressed foreign genes to the next generation. In this study, the production of fertile transgenic tobacco plants which transferred an asulam resistance (*sul* I) gene, the *uidA* reporter gene and the *nptII* (kanamycin resistance) gene to their selfed progeny was confirmed.

The location of foreign gene activity in reproductive structures of transgenic tobacco plants was investigated. In the vegetative structures of transgenic tobacco plants, foreign gene (*uidA*) activity under cauliflower mosaic virus (CaMV) 35S promoter control was highly tissue specific, and was located in the phloem and glandular hairs. In the reproductive structures, this pattern was found to be the same. No CaMV 35S promoter activity could be detected in transgenic pollen.

The regulation of the cauliflower mosaic virus (CaMV) 35S promoter was found to be controlled by factors arising during different developmental phases of the plant. The CaMV 35S promoter was not down-regulated by some *in vitro* applied stress conditions. Anaerobic stress (waterlogging) altered the tissue specific pattern of *uid*A expression under the control of this promoter.

6.1 Introduction

6.1.1 The reproductive processes of dicotyledonous plants. The complex process of sexual reproduction in plants begins with the reorganization of the vegetative shoot apical meristem into the floral meristem (Drews and Goldbery, 1989). The floral meristem produces the reproductive cells - pollen and egg cells (Van Engelen and De Vries, 1992). The reorganization process is initiated by developmental age, photoperiod or temperature (Kelly *et al.*, 1990). Foreign genes introduced into plant somatic cells will also be located in the reproductive cells because of this transition from vegetative to reproductive growth.

6.1.2 Anatomy of the tobacco flower. The structure of a tobacco flower is shown in Fig. 6.1. The internal anatomical structure of the axis of the inflorescence and of the pedicel is similar to that of a typical stem in most plants (Fahn, 1990). Within the receptacle, the shape of the stele follows that of the receptacle. From the stele of the receptacle, the vascular traces lead out to the various floral organs. All the floral organs are vascularized, and the number of traces to the various floral organs varies depending on the plant (Fahn, 1990).

6.1.3 The inheritance of foreign genes in transgenic plants. The outcome of plant genetic engineering experiments must be the production of fertile plants that transmit foreign genes to the next generation. Transmission of foreign genes from one generation to the next depends on transformed cells of the plant becoming ultimately part of the germ line of the plant. In the case of chimaeric plants i.e. plants containing both transformed and non-transformed sectors, foreign gene transfer to progeny may not occur as expected (McHughen and Jordan, 1989). The transformed cells in a chimaeric plant can contribute to the scoring of the plant as transgenic. However, these cells may either not contribute to the germ line (resulting in no transgenic progeny) or contribute to only a portion of the germ line (resulting in many fewer positive segregants than expected) (McHughen and Jordan, 1989). A major aim of plant genetic engineering is to derive non-chimaeric transgenic plants which produce the maximum amount of transformed progeny. The production of fertile regenerated transgenic tobacco derived from

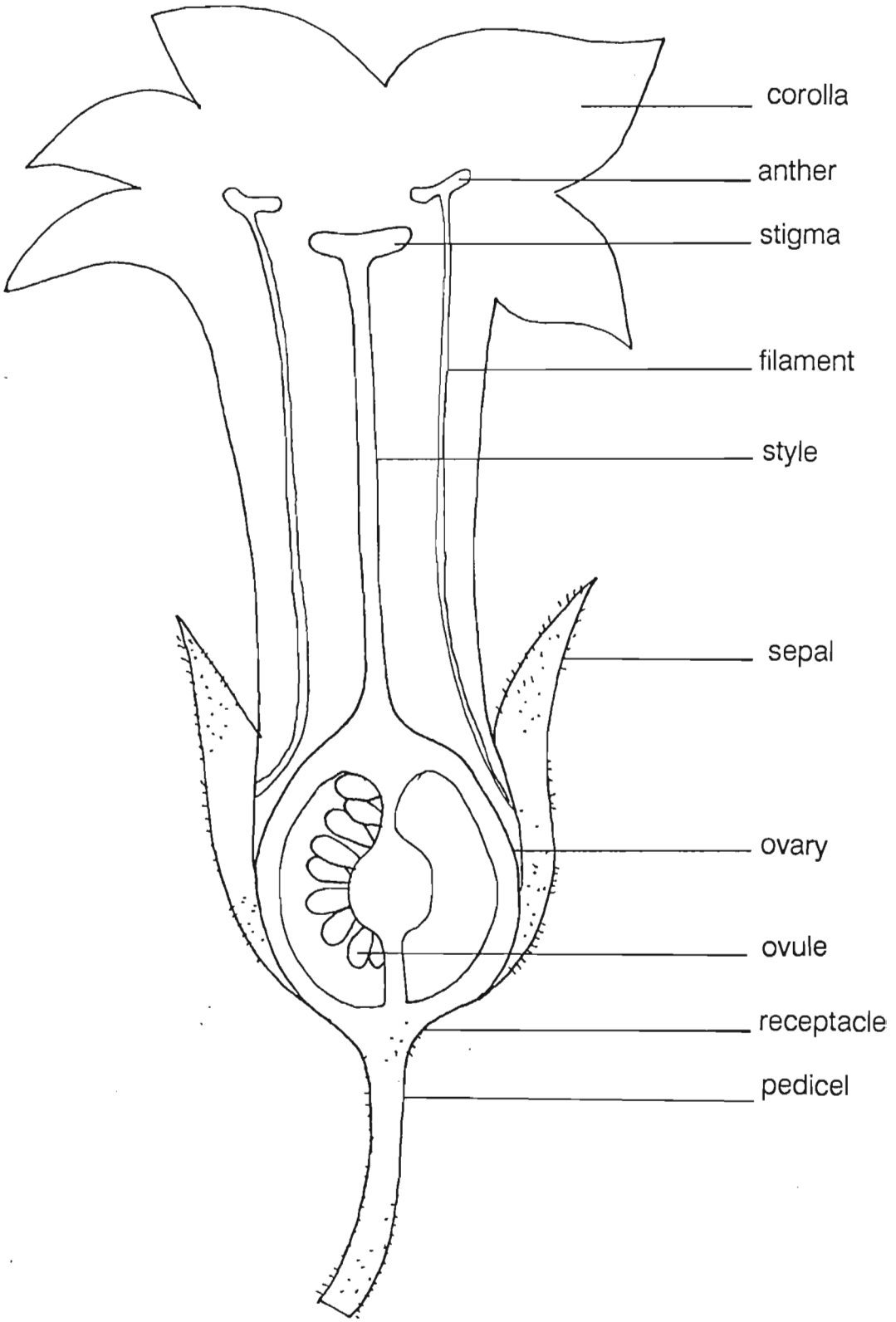


Fig. 6.1. Diagram of a typical tobacco flower

Agrobacterium-mediated transformation is described in the literature, and is usually not problematic (De Block *et al.*, 1984; Horsch *et al.*, 1984; Deroles and Gardner, 1988).

It is important when transgenic plants are to be used for plant breeding that each foreign gene segregates as a single locus (Dunsmuir *et al.*, 1989), and that each foreign gene contains no rearrangements (Deroles and Gardner, 1988). In a breeding programme, repeated selfing of transgenic plants can lead to homozygous plants being obtained which breed true with respect to the foreign gene resulting in transgenic lines (Otten *et al.*, 1981).

Otten *et al.*(1981) also demonstrated a Mendelian pattern of transmission of foreign genes introduced into plants by the Ti plasmids of *Agrobacterium tumefaciens*. In Mendelian inheritance, the segregation ratio of a single dominant gene in a back cross is 1:1 and for a self cross is 3:1. The ratio for two independent dominant genes in a back cross is 3:1 and for a self cross is 15:1. Ratios of lower than 1:1 for a back cross and 3:1 for a self cross indicate unstable inheritance or variable gene control (Deroles and Gardner, 1988). It is also possible that anomolous segregation ratios can arise from a parental plant which was chimaerically transformed. In chimaeric plants, the transformed cells of the plant contribute only a portion of the germ line and this results in fewer positive segregants than expected (McHughen and Jordan, 1989).

Analysis of the segregation ratios of foreign genes in the progeny of self crosses or of back crosses to non-transgenic plants can give information on the gene copy number (Deroles and Gardner, 1988). The analysis of T-DNA segregation in the progeny of transgenic plants, however, does not distinguish between a single gene copy or multiple tandem copies of the T-DNA at the same chromosomal location (Herrera-Estrella and Simpson, 1986).

6.1.4 Tissue specificity of foreign gene expression under CaMV 35S promoter control in transgenic vegetative and floral structures. As there is a complex programme of molecular events which occur in plants during the transition to

flowering , it was thought important to determine whether foreign gene expression under the cauliflower mosaic virus (CaMV) 35S promoter continued in the floral organs of transgenic tobacco plants. The tissue specificity of the *uidA* gene under CaMV 35S promoter control in the floral parts was also investigated. Floral organs of transgenic tobacco plants were sectioned and assayed for *uid*A gene expression to determine whether the pattern of tissue specificity seen in vegetative structures was maintained.

The ability of a foreign gene (*uid*A) to express in the pollen and pollen tubes of a transgenic tobacco plant was also investigated in this study. There is a considerable overlap between genes expressed in the vegetative parts of the plant and simultaneously in the floral structures, including the pollen grains, with at least 60% of the sequences expressed in pollen also expressed in shoot tissues (Mascarenhas, 1990). It is, therefore, reasonable to expect that foreign genes expressed in the vegetative part of the plant could be expressed in the pollen and pollen tube.

6.1.5 Stress and developmental regulation of foreign gene (*uidA*) expression under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Plants, because they are immobile, have developed numerous strategies with which to cope with the many environmental stresses they experience and cannot move away from. The many stresses to which plants are naturally subjected include drought (McCue and Hanson, 1990), salinity (Claes *et al.*, 1990; Jain *et al.*, 1991; Trivedi *et al.*, 1991), aluminium toxicity (Ryan *et al.*, 1992), sulfur dioxide (Madamanchi and Alscher, 1991), heavy metals, chilling (Murata *et al.*, 1992), heat stress (Craig and Gross, 1991) and oxidative stress (Scandalios, 1990).

In order to survive changes in environmental conditions, the plant must be able to respond in a rapid and specific manner by selectively increasing or decreasing the expression of specific genes. As the expression of plant genes is controlled by promoters, any adaptive response involves regulation of promoter activity. For many endogenous plant promoters, activity is regulated by external factors including light (Fluhr *et al.*, 1986), sulphur stress (Rerie *et al.*, 1991), developmental control

(Köster-Töpfer *et al.*, 1989; Rerie *et al.*, 1991), heat (Spena *et al.*, 1985), wounding (Siebertz *et al.*, 1989), phytopathogenic fungi (Roby *et al.*, 1990) or unknown chemical signals (Ward *et al.*, 1993).

A transgenic crop plant would experience the same environmental stresses in the field that a normal plant would experience. It would not be desirable for the expression of an economically important foreign gene to be negatively influenced by these stresses. The nature of *trans* acting factors affecting the cauliflower mosaic virus (CaMV) 35S promoter are not known. It would be important to know to what extent external stress factors experienced by transgenic plants form part of this *trans* acting system.

The CaMV 35S promoter has been the subject of detailed scientific study (Nagata *et al.*, 1987; Benfey *et al.*, 1989; Benfey *et al.*, 1990; Terada and Shimamoto, 1990; Meyer *et al.*, 1992; Dowson *et al.*, 1993: Omirulleh *et al.*, 1993). Like many other promoters functioning in eucaryotes, the expression of the 35S promoter can be regulated in a tissue specific and developmental manner through a complex interaction with *trans* factors present in certain cell types (Benfey *et al.*, 1989; Benfey *et al.*, 1990). The *trans* factors are thought to be promoter binding proteins. The 35S promoter is thought to consist of two domains, each containing a number of *cis*-acting subdomains. These *cis*-elements are able to bind with the cell specific factors in a combinatorial way to achieve varied gene expression (Benfey *et al.*, 1989; Benfey *et al.*, 1990). By different factors acting on the subdomains, the subdomains are able to function independently or synergistically to bring about cell- or organ-specific expression (Benfey *et al.*, 1990). Stress may produce factors which act on the domains of the 35S promoter, affecting expression. Stress conditions experienced by plants could activate a set of endogenous proteins, similar, perhaps to the heat shock response (McKersie and Leshem, 1994).

From a practical point of view, it would be important to determine whether stress could regulate the activity of the CaMV 35S promoter. It would not be desirable for the expression of foreign genes in field grown transgenic plants to be down-regulated by commonly experienced environmental stresses. Similarly, the question

of whether CaMV 35S promoter function was regulated by the developmental stage of the plant, was considered. Many of the tests performed on transgenic plants to determine foreign gene expression are carried out *in vitro*. If foreign gene activity changed as the transgenic plant matured, difficulties may be experienced in selecting transgenic plants based on *in vitro* tests. Greater emphasis would have to be placed on collecting field trial data to assess individual transgenic plants.

The asulam resistance gene (*sul* I) was the major trait of interest and reason for performing genetic engineering on commercial tobacco cultivars. It would be highly undesirable for the activity of this gene under CaMV 35S promoter control to diminish under the stress conditions experienced by mature plants cultivated in the field. The *sul* I gene would not be able to confer asulam resistance on the plants under these conditions.

It would also be undesirable were the high levels of foreign gene expression measured during the *in vitro* stages of plant growth to diminish dramatically as the plant was hardened off and grown to maturity. A brief qualitative study was therefore initiated to investigate the effect of stress and developmental regulation on the 35S promoter in transgenic tobacco transformed with the binary vector pJIT119.

6.2 Materials and Methods.

6.2.1 Inheritance of foreign genes in transgenic tobacco plants. Hardened-off transgenic tobacco plants were allowed to flower. Self-pollination occurred and the plants set seed. The selfed (S_1) seed was used for the analysis of foreign gene inheritance. The seed from the transgenic tobacco plants numbered 2, 3, 5, 6, 7 and 9 were included in the study. Dried seed capsules were collected and stored dessicated at 4°C. To collect the seeds from the pods, the top of each capsule was carefully cut away and the seeds poured into a sterile bottle. Care must be taken not to contaminate the seeds with fragmented capsule material. The surface sterilization of the seeds becomes very difficult if this material is present.

To prepare the transgenic seeds for analysis, the seeds were surface sterilized in 1% sodium hypochlorite containing 0.01% Tween 20 for 30 minutes. The seeds were rinsed twice in sterile distilled water after the sterilization treatment. Using a small syringe, between 100 - 200 seeds were distributed evenly over the surface of the germination medium. The germination medium was based on MS salts and vitamins, with 3% sucrose (Murashige and Skoog, 1962). Germination medium was made without additives, or contained either 100 mg/l kanamycin or 150 mg/l asulam.

After six weeks of culture under a 16 hour day length at 26°C, seedlings with a sensitive or resistant genotype became evident. Asulam or kanamycin sensitive seedlings were bleached and did not produce leaves. Resistant seedlings in this study could be either green (kanamycin) or bleached (asulam), and produced leaves. The ratio of kanamycin or asulam sensitive seedlings to kanamycin or asulam resistant seedlings was determined by counting individual seedlings. Untransformed, tobacco seeds of cultivars TL33 and J6 were also germinated on each type of medium. A ß-glucuronidase (GUS) histochemical assay was performed on seedlings of transgenic plants 5 and 9 (parental plants both GUS positive). To view the GUS colour reaction clearly, the chlorophyll was removed from the seedlings by immersion in 70% alcohol for two hours at 30°C.

6.2.2 Tissue specificity of foreign gene (*uidA*) expression under CaMV 35S promoter control in transgenic tobacco vegetative and floral structures.

6.2.2.1 GUS histochemical assay of vegetative structures. Stem and leaf midribs of transgenic plants were sectioned by hand and subjected to the GUS histochemical assay (Jefferson, 1987). The sections were viewed under the light microscope and ß-glucuronidase activity (blue colour) in the sections recorded photographically.

6.2.2.2 GUS histochemical assay of floral parts. Transgenic tobacco flowers were carefully dissected. The pedicel, receptacle and other floral organs were sectioned transversely by hand. The sections were immediately placed in a 0.01M solution of boric acid for 20 minutes to combat phenolic browning, before being assayed for GUS activity according to Jefferson (1987). Incubation was at 37°C overnight. The sections were examined and photographed using a Zeiss C35 photomicroscope.

6.2.2.3 GUS histochemical assay of pollen. Fresh pollen grains collected from newly opened flowers of plant 9 were assayed using the GUS histochemical method of Jefferson (1987). Pollen grains also were germinated at 30°C for 8 hours in a liquid MS medium containing 3% sucrose. Following germination and pollen tube elongation, the pollen was assayed for GUS activity. MIcroscopic examination and photography was carried out using a Zeiss C35 photomicroscope.

6.2.3 Stress and developmental regulation of foreign gene (*uidA*) expression under CaMV 35S promoter control in transgenic tobacco plants.

6.2.3.1 Analysis of stress regulation of foreign gene (*uidA*) expression under CaMV 35S promoter control. The ß-glucuronidase (GUS) reporter gene was used to study the effect of stress on foreign gene expression under the control of the CaMV 35S promoter in transgenic tobacco. Transgenic GUS positive plants were grown from seed to provide material for this study. The transgenic seed was collected from plant 9 following selfing.

To determine the effect of environmental stresses on forgeign gene (*uidA*) activity, an *in vitro* assay system was used. Leaves from GUS positive greenhouse grown transgenic plants were removed and surface sterilized. Small squares (1 cm^2) of leaf were placed on shoot inducing medium containing additives resembling stress components found in the environment. These included sodium chloride (0.2 M, 0.3 M, 0.4 M and 9.0 M), mannitol (0.4 M, 0.5 M, 0.7 M and 1.0 M) and aluminium chloride (20 μM, 50 μM, 100 μM, 150 μM and 300 μM). The leaf squares were cultured under a 16 hour daylength at 26°C, in a plant growth room. The light intensity was 62.5 $\mu E\ m^{-2}\ s^{-1}$. After ten weeks, the leaf squares were assayed for *uid*A expression using the histochemical GUS assay (Jefferson, 1987).

To test the effect of nutrient stress on foreign gene (*uidA*) expression, leaf pieces were placed on a depleted shoot inducing medium (1/20 MS salts and vitamins, 2 g/l sucrose) for ten weeks. At the end of this period, the leaf squares were assayed for GUS activity according to Jefferson (1987).

To test the effect of anaerobic conditions on *uid*A expression, 2 cm lengths of transgenic tobacco stems were placed lengthways in shallow distilled water in tissue culture bottles, and cultured in the light at 26°C. In this system, one half of the stem segment was submerged, while the other half was exposed to air. After two weeks, the stem sections were removed from the water, sectioned transversely by hand and the GUS histochemical assay carried out.

6.2.3.2 Developmental regulation of foreign gene (*uidA*) expression under CaMV 35S promoter control. To test ß-glucuronidase activity during different phases of plant growth, including *in vitro* culture, the GUS histochemical assay was used (Jefferson, 1987). Clonally multiplied plants of transgenic plant 9 were prepared through nodal propagation so that identical material could be tested under different conditions. Some of these plants were maintained *in vitro* while others were hardened off and grown under greenhouse conditions. Roots and midribs of both *in vitro* and hardened off plants were analyzed using the ß-glucuronidase (GUS) assay. Leaf material from both *in vitro* and hardened off plants was also placed on shoot inducing medium to under go shoot organogenesis. After four weeks, the

resulting shoots were assayed using the GUS assay. The plant material was sectioned by hand to facilitate the visualization of *uid*A gene expression.

6.3 Results

6.3.1 The inheritance of foreign genes in transgenic tobacco plants.

Transgenic tobacco plants were allowed to self pollinate and seeds were collected. When the seed was germinated on kanamycin-containing medium, the kanamycin resistant and kanamycin sensitive seedlings segregated according to several expected Mendelian segregation ratios. Table 6.1 shows the segregation ratios of the kanamycin resistance gene (*nptII*) in a number of transgenic tobacco seedlings. The progeny of transgenic tobacco plants numbered 5, 7 and 9 showed a typical 3:1 segregation ration of the *nptII* gene inherited by their progeny. As *nptII* is a dominant gene, this represents a single insertion of a T-DNA molecule containing this gene into the genome of each parental plant (Deroles and Gardner, 1988).

The progeny of tobacco plant 6 were all kanamycin sensitive. Plant 6 passed several early tests for transgenicity, including rooting on kanamycin, but did not transfer an active *nptII* gene to its progeny. An explanation for this observation could be the methylation of foreign genes during seed production.

The progeny of plant 3 showed a segregation ratio of kanamycin resistant to sensitive seedlings of 15:1 indicating that two independent insertions of T-DNA had occurred. Each T-DNA molecule containing an active *nptII* gene. In the progeny of plant 2, a non-Mendelian segregation ratio of 25:1 was recorded. Figure 6.2 shows the reaction of kanamycin resistant and kanamycin sensitive progeny seedlings on germination medium containing kanamycin (100 mg/l).

Germinating seedlings from selfed transgenic tobacco plants showed a variety of reactions on asulam-containing medium. The manifestation of asulam sensitivity and asulam resistance were not as clearly defined as those for the *nptII* gene (Fig. 6.3). However, once the asulam resistant and asulam sensitive seedlings had been distinguished, the segregations ratios were Mendelian.

The progeny of transgenic plants 3, 4, 6 and 7 showed no inheritance of the *sul* I

Table 6.1. Segregation ratios of the *nptII* gene in the progeny of selfed transgenic tobacco plants. The germination medium contained kanamycin (100 mg/l).

Plant code	Cultivar	Phenotype of parent	Seedlings counted (resistant: sensitive)	Segregation ratio (resistant: sensitive)
#2	TL 33	sul^r GUS^- kan^r	180: 7	25: 1[a]
#3	J6	$sul^s GUS^-$ kan^r	213: 12	15:1[b]
#5	J6	$sul^r GUS^+$ kan^r	162: 57	3:1[c]
#6	J6	$sul^s GUS^-$ kan^r	0: 88	0:1[d]
#7	Samsun	$sul^s GUS^-$ kan^r	64: 20	3:1[c]
#9	J6	$sul^r GUS^+$ kan^r	115: 32	3:1[c]
Untransformed control	J6	$sul^s GUS^-$ kan^s	0: 100	0:1[d]
Untransformed control	TL 33	$sul^s GUS^-$ kan^s	0: 100	0:1[d]
Untransformed control	Samsun	$sul^s GUS^-$ kan^s	0: 100	0:1[d]

Key: a = anomolous, non-Mendelian ratio
b = two dominant genes (double gene insertion)
c = one dominant gene (single gene insertion)
d = progeny all kanamycin sensitive (no inheritance of *nptII* gene)

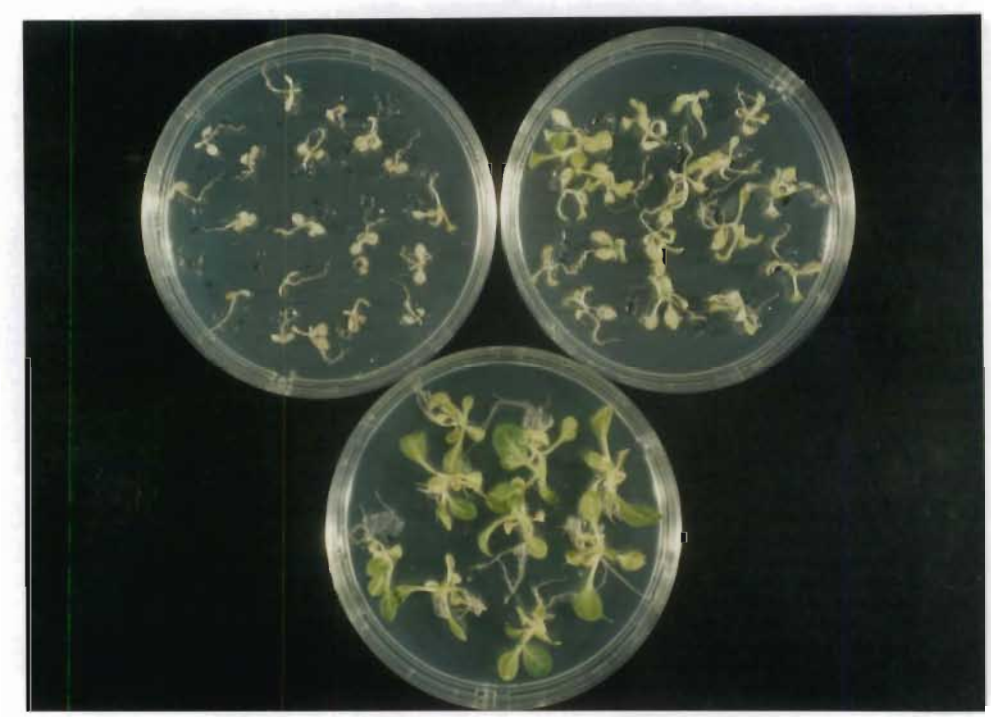
A.

B.

Fig. 6.2. The reaction of kanamycin resistant and kanamycin sensitive progeny germinated on MS medium containing kanamycin (100 mg/l). A) kanamycin resistant seedlings which are green with a well-formed primary leaf pair: B) kanamycin sensitive seedlings which are bleached. Formation of the first leaf pair does not take place.

A.

B.



C.

Fig. 6.3. Transgenic progeny from a selfed asulam resistant transgenic tobacco plant do not show strong resistance to asulam. The seedlings were germinated on MS medium containing asulam (150mg/l). A) asulam sensitive seedlings; B) bleached, stunted, asulam resistant seedlings; C) seedlings grown on asulam-free medium showing normal seedling size and colour.

gene. This was to be expected as their phenotypes (asulam sensitive) (Tables 4.1 and 6.2) indicated that they were all sul^s. All the progeny of these plants died soon after germination on medium containing asulam.

Progeny of plant number 5 showed a typical 3:1 segregation ratio of the *sul* I gene, once the sensitive and resistant seedlings had been distinguished. The asulam sensitive seedlings died soon after germination. The asulam resistant seedlings ranged in appearance from very small (0.5 cm tall) and green in colour, to large (1.0 cm tall) and green in colour. When seeds of this transgenic plant were germinated on MS medium without asulam, the seedlings were large (1.5 cm tall) and green in colour.

The progeny of transgenic plant 9 showed a 3:1 segregation ratio of resistant to sensitive seedlings. The asulam sensitive seedlings died soon after germination. The resistant seedlings were not fully protected against asulam. Although they were green, they were very small (0.5 cm tall). When seedlings of this plant were grown on medium without asulam, normal 1.5 cm tall, green seedlings resulted.

The progeny of plant 2 also showed a variety of reactions on asulam. Asulam sensitive seedlings were identified which survived on asulam, but were stunted and bleached. The asulam resistant seedlings were also bleached and stunted, but they were slightly bigger than the asulam sensitive seedlings. When seedlings from this plant were germinated on asulam-free medium, they were green and of normal size (1.5 cm tall). Despite the similarity between the different types of progeny, the segregation ratio of the *sul* I gene in the progeny of plant 2 was 3:1. The segregation ratios for the *sul* I gene are shown in Table 6.2.

Of the adult transgenic plants, only plants 5 and 9 expressed the *uidA* gene. Progeny of plants 5 and 9 were analyzed for GUS expression. In the selfed progeny of plant 5, a 3:1 segregation ratio of the *uid*A gene was found. It was also confirmed that the *uid*A and *nptII* genes were coinherited i.e. green kanamycin resistant seedlings were GUS positive and bleached kanamycin sensitive seedlings were GUS negative. A single T-DNA insertion had, therefore occurred, and both *uid*A and *nptII*

Table 6.2. Segregation ratios of the *sul* I gene in the progeny of selfed transgenic tobacco plants. The seedlings were germinated on asulam containing medium (150 mg/l).

Plant code	Cultivar	Phenotype of parent	Number of seedlings counted	Segregation ratio (resistant: sensitive)
#2	TL 33	sul^rGUS^- kan^r	68: 23	3:1[a]
#3	J6	sul^sGUS^- kan^r	0: 120	0:1[b]
#5	J6	sul^rGUS^- $nptII^r$	24: 8	3:1
#6	J6	sul^sGUS^- kan^r	0: 150	0:1
#7	Samsun	sul^sGUS^- kan^r	0: 150	0:1
#9	J6	sul^rGUS^+ kan^r	224: 75	3:1
#11	TI 33	sul^sGUS^- kan^r	0: 150	0:1
#4	TI 33	sul^sGUS^- kan^r	0: 200	0:1
Untransformed control	TL 33	sul^sGUS^- kan^s	0:100	0:1
Untransformed control	J6	sul^sGUS^- kan^s	0: 100	0:1
Untransformed control	Samsun	sul^sGUS^- kan^s	0:100	0:1

Key: a = one dominant gene (single gene insertion)
b = progeny all asulam sensitive (no inheritance of *sul* gene)

genes were located on this T-DNA molecule.

The progeny of plant 9, when analyzed, fell into three phenotypic classes when cultured on medium containing kanamycin. The first class included kanamycin resistant seedlings which germinated normally on medium containing kanamycin and which were GUS positive. Two classes of kanamycin sensitive progeny were seen. A group of kanamycin sensitive progeny were found to be GUS positive. Another class of kanamycin sensitive progeny were found to be GUS negative (Fig. 6.4).

In the progeny of plant 9, a 3:1 ratio for the inheritance of *nptII* was indicated. However, the *uid*A gene was inherited in a 15:1 ratio. It had often been noted that transgenic plant 9 expressed the GUS gene more strongly than the other GUS positive plant, plant 5. The segregation ratio of 15:1 confirmed that the strong GUS expression was probably caused by an extra copy of this gene integrated into the genome. Two copies of the *uid*A gene would result in a greater amount of ß-glucuronidase produced if both copies were expressed. These results are shown in Table 6.3. The results show that there are two separate T-DNA inserts in the genome of plant 9. However, in one copy of the T-DNA molecule, the *nptII* and *sul* I genes are inactive. In the other copy of the T-DNA molecule, the *uidA*, *nptII* and *sul* I genes are all active. If all genes on both T-DNA molecules were active, a 15:1 ratio of inheritance of the *uidA*, *sul* I and *nptII* genes would have be found.

6.3.2 Tissue specificity of foreign gene (*uidA*) expression under CaMV 35S promoter control in transgenic vegetative and floral structures.

6.3.2.1 GUS assay of vegetative organs. GUS activity in stem sections of transgenic tobacco (plant 9) was located in the internal and external phloem, and glandular hairs (Fig. 6.5). The pattern of GUS expression in the leaf midrib of a transgenic tobacco plant was similar to that found in the stem. The pattern of distribution of foreign gene activity in transgenic tobacco sections was confirmed using *in situ* hybridization (Chapter Five). This tissue specific pattern of foreign gene expression in the vegetative tissues was compared with the location of foreign gene expression in the floral tissues.

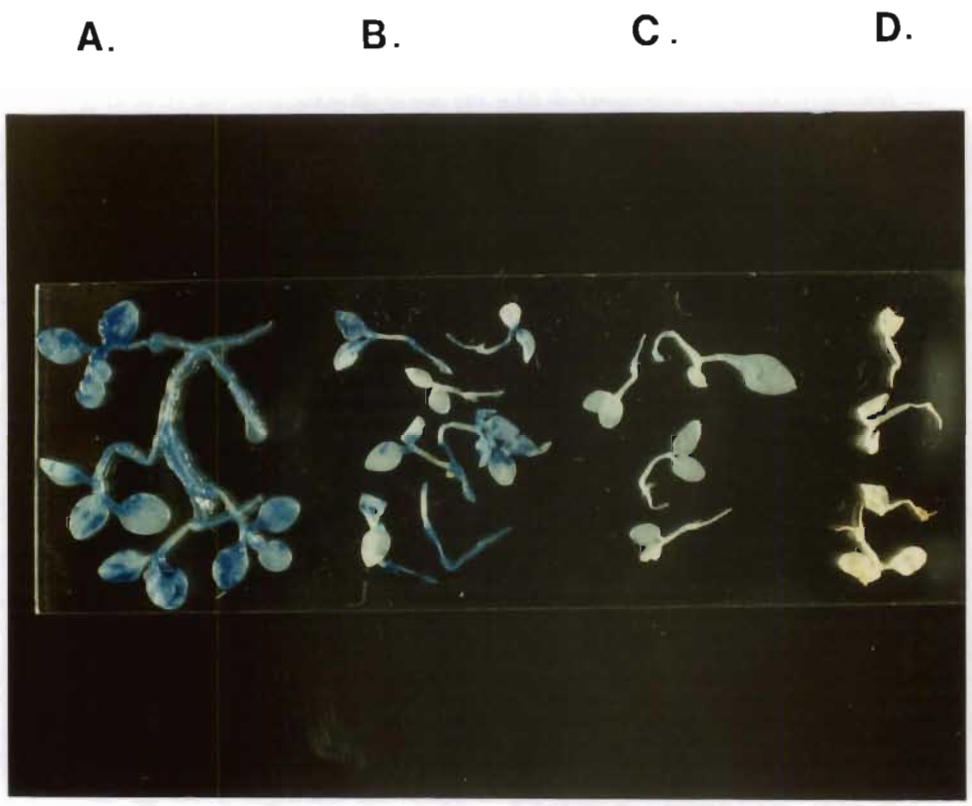


Fig. 6.4. ß-glucuronidase (GUS) assay of transgenic progeny from selfed transgenic tobacco plant 9 showing the inheritance of the *uidA* gene in kanamycin sensitive progeny. A) kanamycin resistant, GUS positive progeny; B) kanamycin sensitive, GUS positive progeny; C) kanamycin sensitive, GUS negative progeny; D) untransformed seedlings. The seedlings were all germinated on tissue culture medium containing kanamycin (100 mg/l). The blue colour shows the activity of the ß-glucuronidase enzyme.

Table 6.3 Segregation ratios of the *uid*A gene in the progeny of selfed GUS positive transgenic tobacco plants.

Plant code and cultivar	Phenotype of parent	Phenotype of progeny[a]	Segregation ratio of *uid*A gene (GUS positive: GUS negative)
# 5 J6	sul^rGUS^+ kan^r	Kan^sGUS^- kan^rGUS^+	3: 1
# 9 J6	sul^rGUS^+ kan^r	kan^sGUS^+ kan^rGUS^+ kan^sGUS^-	15: 1

[a] = Selection carried out on kanamycin (100 mg/l).

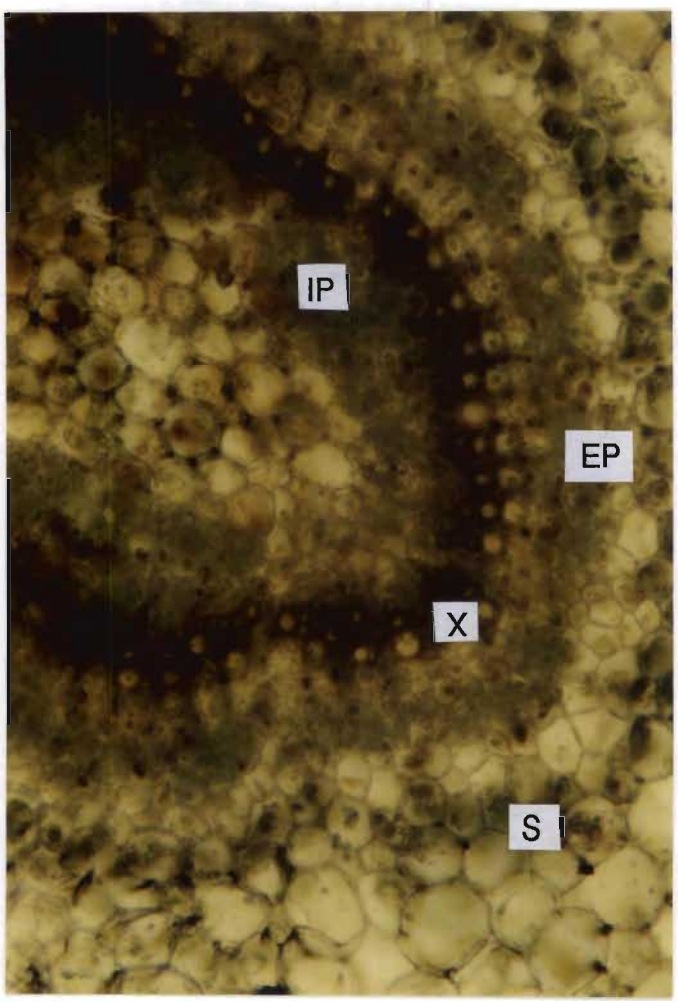


Fig. 6.5. Stem section of transgenic tobacco plant. The location of *uidA* gene expression within the external and internal phloem can be seen (blue colour). IP = internal phloem; EP = external phloem; S = starch sheath; X = xylem (x 1750).

6.3.2.2 GUS assay of floral organs. All sections of floral organs showed GUS activity. The *uid*A gene expression was associated with the vascular tissues and glandular hairs of each organ. The tissue specificity of foreign gene expression under the control of the 35S promoter in reproductive tissues was, therefore, the same as in vegetative tissues.

The entire flower is vascularized, with each organ containing a single, central vascular bundle. The ovary has a different pattern of vascularization, with the ovary containing two large central vascular bundles. The pedicel of a tobacco flower has the same internal structure as a tobacco stem. A central vascular bundle made up of internal and external phloem was present. Strong *uid*A gene expression was associated with the internal phloem of the pedicel, while the *uid*A gene expression in the external phloem was weak (Fig. 6.6). The glandular hairs also showed some GUS activity. No *uid*A gene expression was seen in the cortex, pith or epidermis of the pedicel.

The receptacle is a region of transition for the vascular system between the central stele of the stem and the vascular bundles leading to each floral organ. The receptacle possessed *uid*A gene expression located around the vascular traces (Fig. 6.7).

Transversely sectioned petals also showed a positive GUS reaction associated with the vascular traces. Transverse sections of the filament of the stamens also showed a positive GUS reaction associated with the central vascular trace (Fig. 6.8). Sections of the ovary showed a positive GUS reaction which was associated with the two central vascular traces (Fig. 6.9). At the base of each ovule within the ovary, GUS gene activity was noted in the region of the vascular supply to each ovule (Fig. 6.10).

6.3.2.3 GUS histochemical assay of pollen. Pollen grains collected from 20 transgenic anthers showed no GUS activity (Fig. 6.11). Pollen grains were successfully germinated in liquid MS medium and substantial pollen tubes developed. None of the germinated pollen grains or pollen tubes expressed the *uid*A

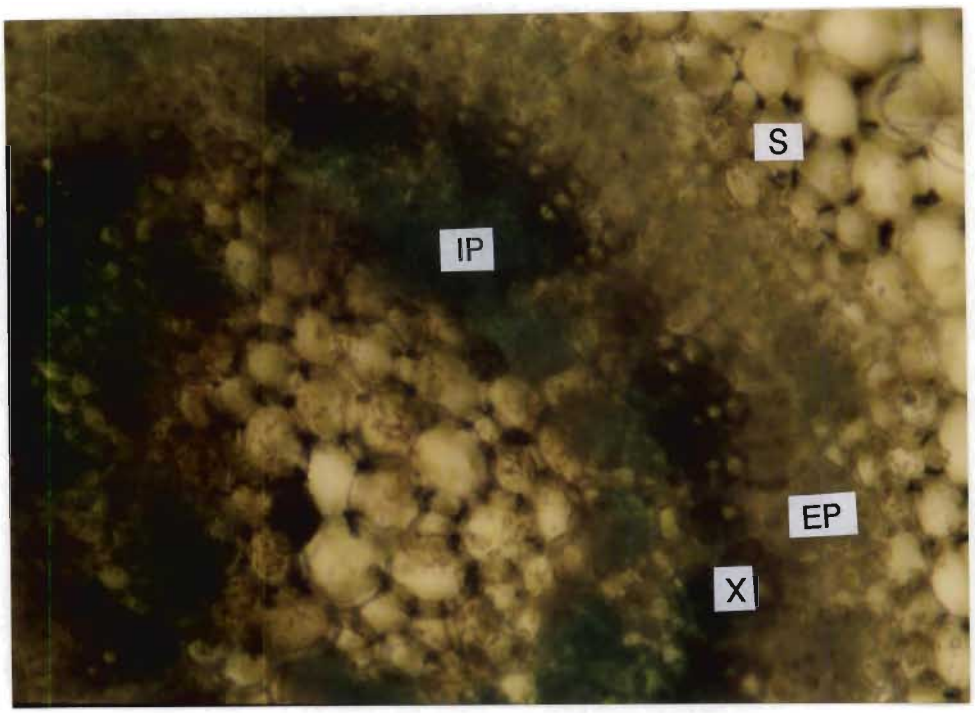


Fig. 6.6. Section of a pedicel from a transgenic tobacco flower showing *uidA* gene expression located mainly within the internal phloem. The activity within the external phloem is very weak. IP = internal phloem; EP = external phloem; S = starch sheath; X = xylem (x 1750).

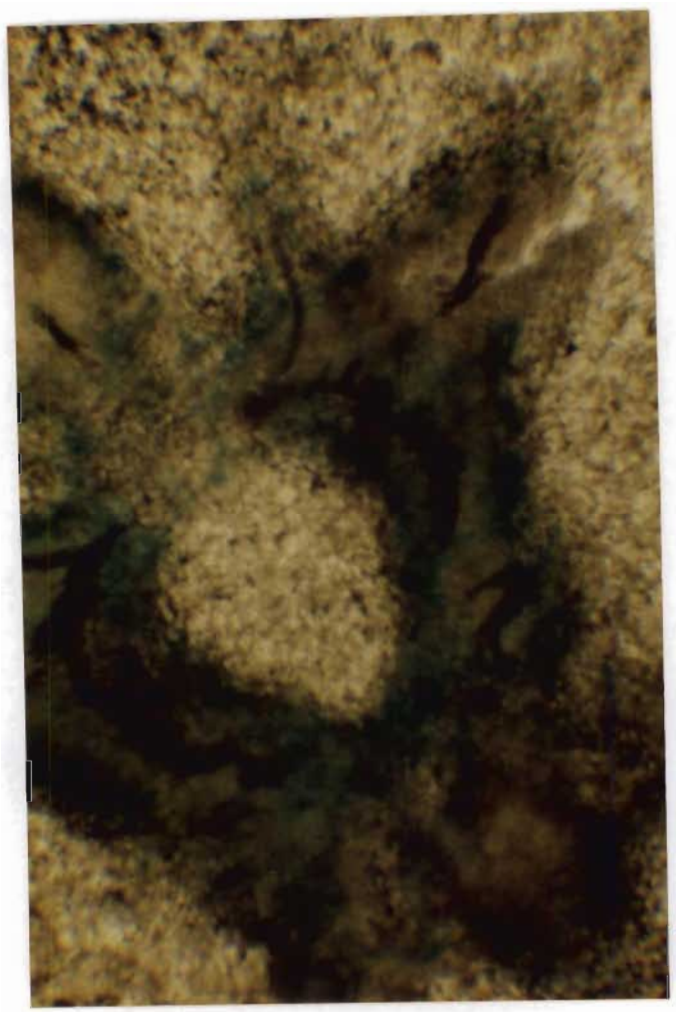


Fig. 6.7. Section of a mature receptacle of a transgenic tobacco flower. The blue colour associated with *uidA* gene expression is located around the vascular traces leading to petals, sepals and other floral organs (x 870).

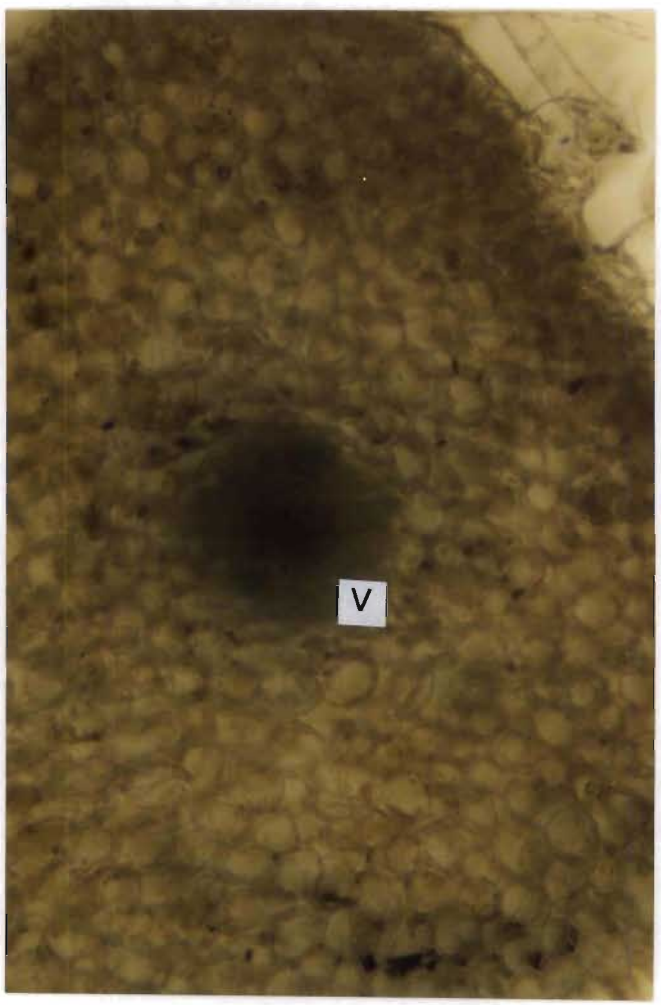


Fig. 6.8. *UidA* (GUS) reporter gene expression associated with the vascular region of a stamen. The *uidA* gene expression is located in the area of the central vascular bundle. V = vascular bundle (x 1750).

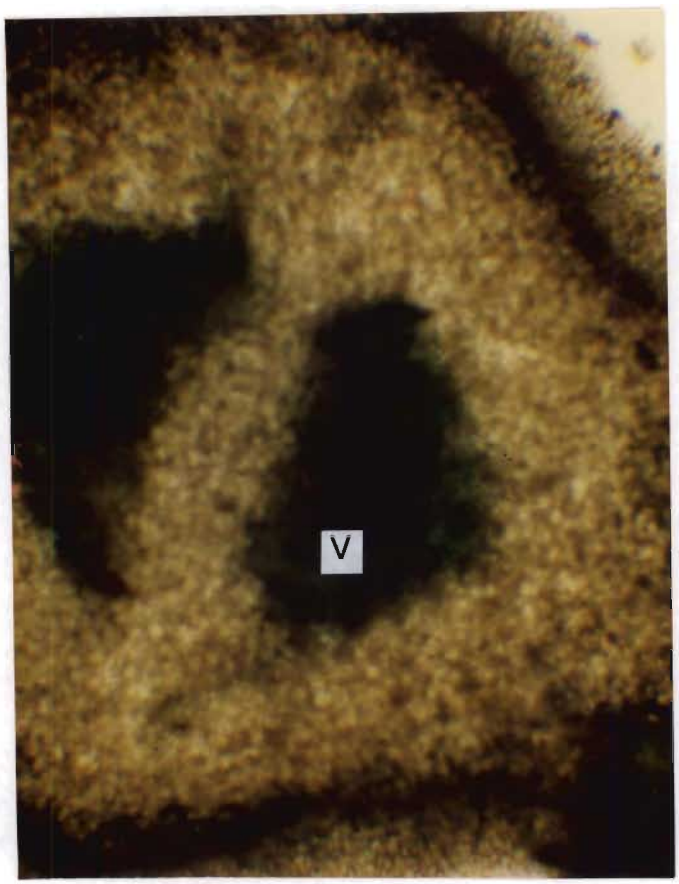


Fig. 6.9. *UidA* (GUS) gene expression in a transgenic tobacco ovary. *UidA* gene expression is located in the area of the vascular traces of the ovary of a transgenic tobacco flower. The main vascular bundles are in the placental region of the ovary. V = vascular bundle (x 280).

Fig. 6.10. Ovules of a transgenic tobacco flower showing *uidA* gene expression. The blue colour (arrowed) associated with *uidA* gene expression was located at the base of each ovule where the vascular tissue supplies each ovule (x 870).

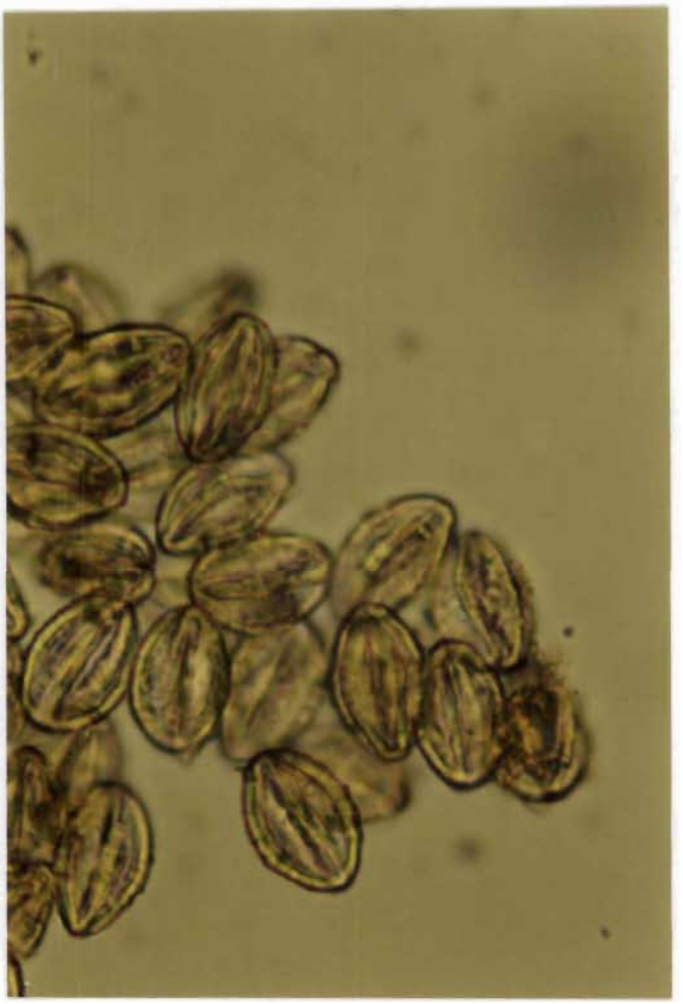


Fig. 6.11. Mature pollen grains from a transgenic tobacco flower did not express the *uidA* reporter gene and show no blue colour associated with ß-glucuronidase activity (x 1750).

gene (Fig. 6.12).

6.3.3 Stress and developmental regulation of foreign gene expression in transgenic tobacco plants.

6.3.3.1 Stress regulation of foreign gene expression. The experiment to test the effects of *in vitro* stress treatments of sodium chloride, mannitol and aluminium chloride on ß-glucuronidase gene *(uid*A) expression in leaf samples of transgenic plants, resulted in two categories of leaf explants after each of the treatments.

The first category consisted of leaf pieces which were still green after being cultured on the lower levels of NaCl, mannitol and $AlCl_2$ for ten weeks. The second category consisted of leaf samples which were severely bleached after ten weeks culture on the higher levels of NaCl, mannitol and $AlCl_2$.

The GUS assay performed on the first category of leaf pieces showed that these leaf pieces expressed strong GUS activity (data not shown). However, a GUS assay performed on the transgenic leaf samples in the second category yielded unexpected results. It had been assumed, following a visual inspection of the leaf pieces in this category, that the leaves were too damaged by the chemicals included in the medium to express GUS. However, out of a random selection of twenty five leaf pieces, twenty expressed moderate to strong GUS activity (Fig. 6.13).

After ten weeks on a depleted shoot inducing MS medium, a visual examination showed that the effect of nutrient deprivation on leaf pieces of transgenic tobacco plants was extreme bleaching. Adventitious shooting had occurred initially, but the shoots had bleached once the nutrients in the medium were used up. Random samples of material taken for GUS histochemical assay showed weak to moderate ß-glucuronidase activity (Fig. 6.14). Brown and dead tissue did not express GUS activity.

As a result of oxygen deprivation through waterlogging, the tissue specific pattern of *uid*A gene activity under CaMV 35S promoter control was found to have changed.

Fig. 6.12. Germinated pollen grains and pollen tubes from a transgenic tobacco flower did not express the *uidA* reporter gene. There is no evidence of the blue colour associated with ß-glucuronidase activity (x 1750).

Fig. 6.13. Results of a ß-glucuronidase (GUS) histochemical assay on chemically stressed transgenic tobacco leaves. Transgenic tobacco leaf squares were subjected to a variety of chemical stress conditions *in vitro*. A random selection of those leaf samples showing severe signs of stress under the experimental conditions were assayed. The leaf samples expressed the *uidA* reporter gene despite their poor condition.

A.

B.

C.

Fig. 6.14. ß-glucuronidase (GUS) assay of transgenic tobacco leaf samples subjected to nutrient deprivation *in vitro*. A) Petri dish containing leaf pieces with adventitious shoots which have become bleached after ten weeks culture on a depleted MS medium; B) and C) GUS histochemical assay of nutrient stressed leaf samples and adventitious shoots. Most of the nutrient stressed samples expressed ß-glucuronidase activity.

The side of the stem segment, which had been under water for two weeks had strong GUS activity in the epidermis and cortex, instead of only in the phloem and glandular hairs as is normally seen (Fig. 6.15). This unusual GUS activity was located towards the side of the stem which was under the water i.e. the oxygen-deprived section.

6.3.3.2 Developmental regulation of foreign gene expression. An investigation into the developmental effects on foreign gene expression showed evidence of regulation of the *uidA* gene under the control of the 35S promoter in transgenic tobacco. Leaf, root and stem samples taken from *in vitro* transgenic plantlets expressed ß-glucuronidase at much higher levels than equivalent samples taken from mature transgenic plants grown in the greenhouse (Fig. 6.16).

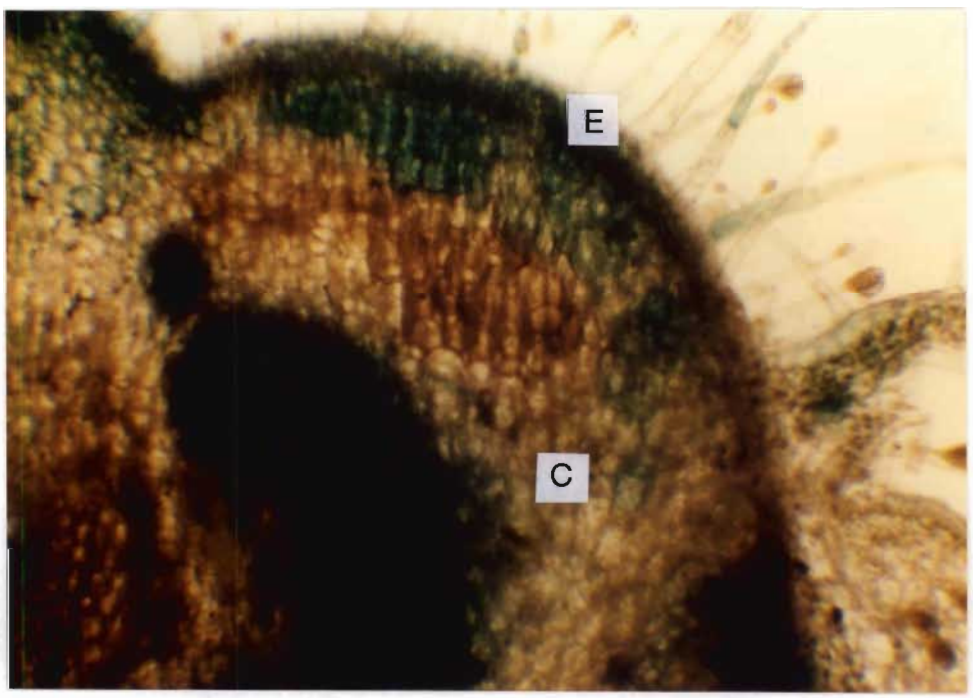


Fig. 6.15 Section of transgenic tobacco stem segment subjected to oxygen deprivation through waterlogging. The GUS activity is seen in the cortex (C) and epidermal cells (E), instead of only in the external and internal phloem, and glandular hairs (X550).

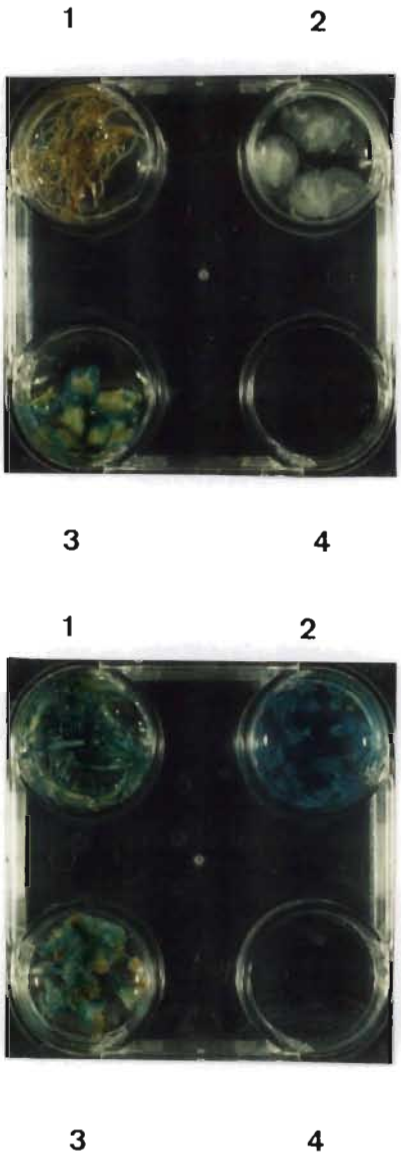


Fig. 6.16. Developmental regulation of the *uidA* gene under the control of the CaMV 35S promoter in transgenic tobacco. The samples from *in vitro* grown plants express stronger ß-glucuronidase (GUS) activity than mature, greenhouse grown plants. A) Mature transgenic tobacco material assayed for GUS activity. Well 1, roots; well 2, stem sections; well 3, leaf pieces undergoing adventitious shoot production; B) transgenic tobacco material grown *in vitro* and assayed for GUS activity. Well 1, roots; well 2, stem and midribsections; well 3, leaf pieces undergoing adventitious shoot induction.

6.4 Discussion

In assessing the value of individual transgenic plants, it is critical to determine whether they are fertile and able to transmit foreign genes to their progeny (McHughen and Jordan, 1989). This indicates that stable transformation has taken place (Deroles and Gardner, 1988). Seedling progeny from selfed transgenic tobacco plants were found to have inherited foreign genes from the parental plants. Examination of the segregation ratios of the foreign genes also provided an estimate of the gene copy number (De Block *et al.*, 1984; Deroles and Gardner, 1988).

Examination of the segregation ratios of the kanamycin resistance gene (*nptII* gene), showed normal Mendelian segregation ratios of 3:1 (plants 5, 7 and 9) and 15:1 (plant 3). In one case (plant 2) an anomolous ratio of 25:1 was found. Deroles and Gardner (1988) studied the inheritance patterns of transgenic petunia plants in detail to obtain a spectrum of segregation ratios for the *nptII* gene. As well as groups of transgenic progeny falling into normal segregation classes, a group of seedlings which had "anomolous" ratios unlike any Mendelian patterns was found in their study. The progeny of plant 2 fall into this "anomolous" class of inheritance.

The progeny of transgenic plant number 6 showed a segregation ratio of 0:1, i.e all the progeny were kanamycin sensitive. The parental plant was kanamycin resistant. This could occur if the foreign genes in this transgenic plant had undergone methylation during seed formation (Deroles and Gardner, 1988).

In the test for transmission of the asulam resistance (*sul* I) gene to the progeny of transgenic tobacco plants, segregation ratios of 3:1 were obtained. However, the inheritance patterns were complicated by variation in the sensitivity and resistance reactions of the progeny seedlings. The asulam resistant progeny seedlings of plant number 5 were different sizes. In the collection of seeds for experimentation, seeds collected from many capsules of each individual plant were pooled. Although, in a self-cross all progeny are almost identical, meiosis during gamete formation does allow genetic variation to occur. This could explain the variable progeny. Deroles and Gardner (1988) also obtained progeny with variable inheritance patterns from

different seed capsules located on the same plant.

The asulam sensitive progeny of plant 2 did not behave as expected. They did not die on asulam containing medium as asulam sensitive progeny from other selfings did. The asulam sensitive progeny from plant 2 germinated, but were bleached and stunted. The asulam resistant progeny from this plant were also bleached, but grew to a normal size. Progeny of transgenic plants with reduced levels of resistance when compared to the parent plant can be expected due to some down regulation of gene expression during plant development (Deroles and Gardner, 1988). However, it is not clear why the asulam sensitive progeny of plant number 2 survived better on asulam containing medium than control plants of this cultivar (TL33). The cultivar has no endogenous resistance to asulam.

In this study, parental transgenic tobacco plants which were asulam sensitive produced only asulam sensitive progeny. The parental plants had previously been shown to contain the *sul* I gene, but in a rearranged form (Chapter 5). This inactive form of the *sul* I gene would also be transmitted to the progeny, along with the other progeny foreign genes, in a 3:1 ratio. There was no reactivation of the *sul* I gene in the progeny. It would be possible for transgenic parental plants not expressing a foreign gene to produce progeny expressing the foreign gene. This would occur, for example, if selectively methylated foreign genes became demethylated during seed production (Deroles and Gardner, 1988).

Foreign gene inactivation was evident in plant number 9. The *nptII* and *sul* I genes were inherited in a 3:1 ratio indicating a single foreign gene insertion into the genome. The *uidA* gene was inherited in a 15:1 ratio, indicating two independent T-DNA insertions into the plant genome (Fig.6.12). The *nptII* and *sul* I genes on one of the T-DNA molecules have been inactivated. Also, in plant 3, a similar inactivation had occurred. In the progeny of this plant, a segregation ratio of 15:1 (Table 6.1) for the *nptII* gene showed that on two independent T-DNA insertions, both copies of *uidA* and *sul* I genes were inactive, while the *npt*II gene on each T-DNA insert was functional.

The data gathered for this study showed conclusive evidence that the all transgenic plants obtained in this study were stably transformed and fertile. The transgenic tobacco plants analyzed were all able to transmit foreign genes to progeny, which confirms stable transformation and fertility. The transgenic tobacco lines produced in this study could be included in a breeding programme for tobacco improvement.

Following on from a study of the inheritance of foreign genes by transgenic selfed progeny was an investigation into the spatial pattern of foreign gene (*uidA*) expression in the reproductive structures of transgenic tobacco flowers.

A critical developmental event in higher plants is the transition from vegetative to reproductive growth, marked by the onset of flowering (Drews and Goldberg, 1989). Changes in RNA and protein synthesis occur during this transition, indicating that modulation of gene expression is taking place. New genes which are not active in the vegetative plant are expressed in the reproductive structures. Additionally, many of the mRNA species which are found in the mature vegetative plant are also found during floral initiation and subsequent floral development (Meizer *et al*., 1989).

Of the floral parts, the floral organs that most closely resemble leaves are the sepals. The petals are also similar to leaves, but are pigmented. The stamens and carpels differ greatly in appearance from leaves, yet commonly accepted evolutionary theory on their development is that they are also homologous to leaves (Fahn, 1990). It is not difficult to envisage that the tissue specific pattern of foreign gene expression in vegetative parts of the plant, including stems and leaf midribs, will continue into the floral tissues and organs.

The tissue specific pattern of *uidA* gene under CaMV 35S promoter control in floral parts of the transgenic plant material analyzed was found to be essentially the same as that found in stems and leaves of the vegetative plant. *UidA* gene activity was associated with the vascular tissue and glandular hairs in vegetative and floral structures. On the whole, the pattern of *uidA* expression associated with the vascular tissue of the reproductive parts of the plant was preserved even in the very small vascular bundles of the filaments, petals and style, while the *uidA* expression at the

base of the developing ovules could also be explained as being associated with a vascular supply to each ovule. In the pedicel of transgenic tobacco flowers, however, the *uidA* expression was found to be stronger in the internal phloem of the vascular tissue than in the external phloem.

Gene expression during stamen development, microsporogenesis and pollen germination has been well studied (Mascarenhas, 1990; Gasser, 1991). There is an extensive genetic programme expressed during pollen development with up to 24 000 different mRNAs being found in developing and mature pollen (Mascarenhas, 1990). However, most genes transcribed and translated during pollen development are "housekeeping genes" also expressed in vegetative parts of the plant, with the overlap estimated at 60-65% (Gasser, 1991; Frova, 1990). Good correlation therefore should exist between characteristics expressed in vegetative tissue and those expressed in pollen, including transgenic characteristics (Bino *et al.*, 1987). However, no *uidA* gene expression could be detected in transgenic pollen. Published studies with electroporated pollen have shown that the CaMV 35S promoter is not expressed in pollen (Fennel and Hauptman, 1992; Van der Leede-plegt *et al.*, 1992). To obtain foreign gene expression in pollen, other pollen-specific promoters must be used (Fennel and Hauptmann, 1992; Van der Leede-Plegt *et al.*, 1992). This is the probable explanation for the lack of *uidA* gene expression in transgenic tobacco pollen. The *uidA* gene used in this study was under the control of a single CaMV 35S promoter. Although the germinating pollen grains did not express the *uidA* gene, they were able to transfer this and other foreign genes to the next generation.

The double CaMV 35S promoters linked to the ß-glucuronidase gene (*uidA*) were not significantly down-regulated in transgenic tobacco tissue during exposure of the tissue to extended chemical, dessicating, oxygen or nutrient stress *in vitro*. Stressed transgenic tobacco leaf material which had become bleached due to prolonged exposure to high levels of aluminium chloride, mannitol or NaCl, or because of extended culture on a dilute MS medium, expressed the *uidA* gene strongly when assayed at the end of the culture period. The half life of the GUS enzyme, B-glucuronidase, is 50 hours in living mesophyll cells (Jefferson *et al.*,1987). Had

exposure of the transgenic leaf material to the stress conditions resulted in an immediate cessation of transcription of the *uidA* gene, or a down-regulation of *uidA* expression, there would be no detectable ß-glucuronidase activity after six or ten weeks. Performing a calculation based on the half life of ß-glucuronidase and a sixteen day period, the cessation of *uidA* expression would result in 1/16 of the original active GUS enzyme remaining in the transgenic tissue after this period of tiem. *UidA* gene activity would hardly be visible at this stage. The transgenic tobacco leaf samples were subjected to stress conditions for far in excess of sixteen days. Strong GUS expression levels at the end of ten weeks appeared to be similar to or greater than those of unstressed tissue. The ß-glucuronidase enzyme active at the end of the experimental period must have been synthesized *de novo* during the period of stress.

While these results would require further testing in mature plants under greenhouse or field conditions, it is encouraging that, at least *in vitro*, foreign gene (*uidA*) expression under the control of the CaMV 35S promoter did not decrease during exposure to stress conditions and in fact, may be enhanced.

In some cases, it was possible for *uid*A activity under CaMV 35S promoter control to increase during stress. Under oxygen limiting conditions, the number of cells within the plant expressing GUS increased. This involved cells which do not normally express GUS (cortex, epidermal cells) to change and express GUS. This change showed that the tissue specificity of *uid*A gene activity under CaMV 35S promoter control was not fixed, and that certain conditions could alter this specificity.

While the 35S promoter appeared to be constitutive during exposure to stress, there was evidence that this promoter was down-regulated by developmental factors as the transgenic plants reached maturity. This has also been noted by Blake *et al.* (1991). During the *in vitro* stages of culture, ß-glucuronidase activity was expressed strongly in roots, stem and leaf midrib sections. This activity decreased dramatically in similar tissues sampled from mature, hardened off transgenic tobacco plants. The original high levels of GUS activity could be restored in mature leaves by placing them back in the *in vitro* environment on shoot inducing MS medium.

The possibility that different levels of foreign gene expression (*uidA*) occur at different stages during transgenic plant production is a potential problem in the development and testing of transgenic plants. Many of the assays for foreign gene activity are performed during the *in vitro* growth phase of the plants. These *in vitro* assays include the callus induction assay, assays for shoot production from leaves cultured on tissue culture medium containing herbicides or antibiotics, the NPTII activity assays, assays for root production from transgenic plants cultured *in vitro* on kanamycin containing medium and computerized image analysis. At all these stages, it appears that the activity of foreign genes under the control of the 35S promoter is higher than that found in mature, hardened off tobacco plants.

Transgenic plants should be tested as mature plants under field conditions rather than *in vitro* in order to select plants which express large amounts of foreign genes. This is the important role of greenhouse and field testing.

This study indicated that the CaMV 35S promoter is regulated in transgenic tobacco tissues. Foreign genes under the control of this promoter show tissue specifity of expression in vegetative and reproductive structures. The activity of this promoter is also controlled by factors arising during different phases of the culture of the plant. This was indicated by the apparent developmental regulation of the CaMV 35S promoter. However, *in vitro* applied stress conditions do not significantly down-regulate the expression of genes under the control of the CaMV 35S promoter and in fact, there may be an enhancement of their continued expression during stress. Certain stress conditions increased the levels of *uid*A expression by altering the tissue specificity of CaMV 35S promoter activity. These results all suggest that the CaMV 35S promoter is a good promoter for commercial production.

Chapter 7

General discussion

In the search for an efficient transformation system to produce transgenic plants of South African tobacco cultivars several approaches were explored; and tobacco cultivars TL33, J6 and 20/19 were transformed for possible agricultural improvement. These cultivars had not previously been genetically engineered in South Africa. Tobacco (*Nicotiana tabacum* L.) is not recalcitrant to regeneration *in vitro* or to transformation, hence the common use of several non-commercial cultivars as model systems in plant genetic engineering studies. To genetically engineer South African tobacco cultivars, tissue culture and transformation methods cited in the scientific literature were employed without modification. No genotype differences were noted in the response of the South African cultivars to *in vitro* manipulation or *Agrobacterium tumefaciens* infection and transformation.

Although the *Agrobacterium*-mediated leaf disc transformation method proved the most successful and trouble-free method to genetically engineer South African tobacco cultivars, both *Agrobacterium*-mediated and direct DNA-mediated methods of genetic engineering were considered and evaluated. Tobacco leaf discs, mesophyll protoplasts and single cell cultures were used as the explant material with which to effect transformation. South African commercial tobacco cultivars TL33, J6 and 20/19 were used as the source of original explant material. Transgenic tobacco plants expressing an asulam resistance gene (*sul* I) were produced using this method. Transgenic, asulam resistant lines of these cultivars now exist.

The preparation of *A. tumefaciens* transconjugant strains containing the plant gene vector pJIT119 (Guerineau *et al.*, 1990) was accomplished using standard methods. Despite warnings that DNA rearrangements can occur during the conjugation process (Draper *et al.*, 1988), this did not happen. The transconjugant strain C58C1(pGV2260)(pJIT119) was stable on antibiotic selection, contained no DNA

rearrangements and was successfully used to produce transgenic tobacco plants in an *Agrobacterium*-mediated leaf disc transformation experiment.

While the transgenic tobacco plants were still in the early stages of *in vitro* culture and selection, plants which had "escaped" kanamycin selection were identified and discarded. It was initially assumed that when "escape" plants occurred, they would possess only an inactive *nptII* gene and that all other foreign genes would be active. When the "non-escape" kanamycin resistant transgenic plants were analyzed in more detail, it was found that "escapes" had occurred in each of the other two foreign genes, *sul* I and *uidA* derived from pJIT119. Were the original discarded "escape" plants analyzed for *uidA* and *sul* I expression, it is highly likely that a portion of these would have been found to be transgenic, expressing the *uidA* and/or *sul* I genes, but having a defective *nptII* gene.

Upon further examination of the group of transgenic tobacco plants, the full extent of their inter-transformant variability was revealed. The variability in foreign gene expression was caused by foreign gene inactivation. All ten transgenic plants studied had one or more non-functional foreign gene derived from the T-DNA of pJIT119. Using *in situ* hybridization and PCR, the cause of this inactivation was deduced to be foreign DNA rearrangements, rather than foreign gene or T-DNA loss. In all ten plants analyzed, the DNA as well as mRNA from the introduced foreign genes was present, regardless of whether foreign gene expression could be detected.

Biochemical assays for NPTII activity, as well as Western blotting, were carried out in order to determine whether these assays could be readily applied to confirm and quantify transformation. The methods involved the use of radioactive compounds (NPTII assays), or fairly lengthy processing (Western blotting). The availability of several assays for the reporter gene ß-glucuronidase (GUS) activity has largely removed the need to perform these other techniques in this context. The spectrophotometric and fluorimetric methods for quantitative ß-glucuronidase assay

(Jefferson, 1987) provide accurate quantitative data on foreign gene (*uidA*) expression. Inter-transformant variability of foreign gene (*uid*A) expression levels in transgenic plants would be accurately determined using these methods.

The use of the image-analyzer was a novel approach used to determine levels of foreign gene (*sul* I) expression in transgenic plants through the measurement of growth of transgenic plant tissue (callus) *in vitro*. The data provided could be graphically displayed, giving a useful representation of the differences in asulam resistance between individual transgenic plants, at different asulam concentrations.

Detailed *in vitro* testing of transgenic plants may or may not be appropriate, depending on the foreign genes transferred into the plants, and their purpose. To save time, transgenic plants containing agriculturally relevent foreign genes should be tested as soon as possible under greenhouse or field trial conditions to determine how they and their novel genes perform. For example, in the case of herbicide resistant plants, spraying with the herbicide in the field will quickly and without elaborate biochemical and molecular assays determine which transgenic plants have full expression of foreign herbicide resistant genes.

However, in order to field test transgenic plants, certain information is required. For example, in Europe, the detailed information that is required for a field release includes revealing the origin and sequence of the transferred DNA. In the transgenic plants, the copy number of the transgene/s, the size of the integrated DNA, and the presence or absence of nonfunctional or unneccessary DNA sequences must be determined. Also, the mode of foreign gene inheritance, the stability of foreign gene expression, the foreign gene expression level in general, the expression levels in pollen (in case of allergic responses) must be known. Also, any pleiotrophic effect of the foreign genes on the plant and its progeny must be determined (G. Donn pers.comm).

In South Africa, the South African Committee For Genetic Experimentation

(SAGENE) has drawn up guidelines for the importation, trial release or general release of genetically modified organisms (GMOs). Although SAGENE has no authority to approve or prohibit the importation, trial or general release of a GMO, it acts as an advisory body to advise Government Departments directly concerned with the release of GMOs (Department of Health, Department of Agriculture, Department of Trade and Industry etc), and to advise organisations wishing to work with or release GMOs. The risk assessment required by SAGENE considers genetic aspects (eg. stability and expression of foreign genes, characterization of the foreign genetic material, effect of foreign gene expression under natural selection, horizontal spread of the genes etc.) as well as ecological issues.

Transgenic tobacco plants are generally fertile and set seed, allowing detailed studies of Mendelian segregation ratios (Draper *et al.*, 1988; Guerineau *et al.*, 1990; Dijak *et al.*, 1991). The inheritance of both the *nptII* and *uidA* genes in the progeny of transgenic tobacco plants produced for this study was easily distinguished in the progeny of the selfed transgenic plants, and was well defined. The inheritance of the *sul* I gene in these progeny was not well defined. In the published report by Guerineau *et al.* (1990), the *sul* I gene behaved as a dominant character and segregated in a normal Mendelian manner. However, the report does suggest that in two plants, the *sul* I gene was only weakly expressed in the progeny. In the transgenic progeny from several asulam resistant tobacco plants which were analyzed in this present study, all expressed the *sul* I gene weakly. These seedlings were scored as asulam resistant, but they had a very similar appearance to the asulam sensitive progeny i.e. they were also bleached and stunted. When the numbers of small bleached or dead seedlings were counted and compared with the numbers of a variety of larger bleached or stunted green seedlings, a 3:1 ratio of asulam resistant to asulam sensitive progeny was evident. One must assume that the small bleached or dead seedlings were truly asulam sensitive, while plantlets which were only slightly larger, but still bleached and/or green were asulam resistant. In this way, the *sul* I gene is an unsatisfactory model gene with which to work. Economically, its value in creating asulam resistant crop lines would also be

questionable because of the poor inheritance of the *sul* I gene by progeny.

However, as an initial *in vitro* selection for transformed plant material in a leaf disc transformation experiment, the *sul* I gene provides a highly effective selection system. Untransformed leaf or callus tissue quickly dies on low levels of asulam *in vitro* (50 mg/l), while transgenic leaf or callus material expressing the *sul* I gene tolerated levels of up to 200 mg/l of asulam *in vitro*.

A number of transgenic tobacco plants became available for further study, providing an opportunity to investigate aspects of cauliflower mosaic virus (CaMV) 35S promoter activity. The cauliflower mosaic virus 35S promoter is a very strong viral promoter, producing the CaMV 35S genomic RNA in infected plants (Kuhlemeier, 1992). The CaMV 35S promoter is not a simple structure, but a complex array of various regulatory *cis*-acting elements (Benfey *et al.*, 1989; Benfey *et al.*, 1990; Kuhlemeier, 1992). It is the sum of all the *cis*-acting elements that results in the constitutive, yet tissue specific activity of this promoter. Some of the genes which code for proteins which bind to the *cis*-acting elements have also been isolated, leading to an understanding of how these elements co-operate with each other to bring about the finely tuned regulation of viral or foreign genes in plants (Kuhlemeier, 1992).

The *sul* I gene from pJIT119 was under the control of a double CaMV 35S promoter, while the *uidA* gene was under the control of a single CaMV 35S promoter. The *uidA* gene expression proved the most convenient to monitor through the GUS histochemical assay. *In situ* hybridization also provided evidence of the tissue specificity of CaMV 35S promoter activity.

The tissue specificity of the CaMV 35S promoter studied by Benfey *et al.* (1989), Benfey *et al.* (1990) and Jefferson *et al.* (1987) was confirmed in this study. As reported, CaMV 35S promoter activity in the transgenic tobacco plants was located in the external and internal phloem cells, as well as glandular hairs of leaves and

stems in transgenic tobacco plants. However, these authors did not study the reproductive organs of transgenic tobacco. In this present study, the tissue specificity of the CaMV 35S promoter was found to extend to the vascular traces of the floral organs and those leading to the developing seeds, as well as in the glandular hairs of the reproductive organs. No *uidA* activity was detected in mature or germinating pollen, indicating that the CaMV 35S promoter activity was so tissue specific that it could not function in the pollen internal environment. However, the pollen would still transmit this and other foreign genes to progeny.

Although in whole transgenic tobacco plants, the CaMV 35S promoter controlling the *uidA* gene had very strong tissue specific activity located only in the phloem cells and cells of the glandular hairs, factors other than the position of cells within the plant must play a role in determining which cells express the *uidA* gene under CaMV 35S control. When callus was made from leaves of GUS positive tobacco plants, the undifferentiated cells expressed the *uidA* gene strongly. However, not all the cells expressed the same intensity of blue associated with *uidA* expression. This differential *uidA* expression could relate to the stage of the cell cycle that the cells are undergoing (Nagata *et al.*, 1987), the different origin of the cells from within the leaf, as well as other unknown factors.

The activity of the CaMV 35S promoter was found to be maintained in transgenic leaf pieces during *in vitro* applied stress conditions. Various authors have reported similar findings (Kay *et al.*, 1987; Odel *et al.*, 1985; Ow *et al.*, 1987; Kuhlemeier, 1992). Reporter genes under CaMV 35S promoter control were always expressed and at high levels, and were insensitive to various endogenous and environmental cues such as hormones, heat shock or light . This characteristic would truly earn the CaMV 35S promoter the reputation for being "constitutive".

Another aspect of CaMV 35S activity to consider is that stress conditions might actually increase foreign gene expression under the control of this promoter, perhaps through post-translational control mechanisms (Kuhlemeier, 1992). This

could also explain the continued strong expression of the *uidA* gene in distressed tobacco leaf pieces after prolonged exposure to *in vitro* chemical and nutrient stress, observed in this present study. These observations would indicate that under field conditions, transgenic plants with foreign genes under CaMV 35S control might continue expressing these foreign genes at normal or even increased levels when exogenously applied stresses are experienced. This is an important finding, as it would be undesirable for foreign gene activity, introduced into plants at great cost, to disappear in field grown plants during normally experienced stresses. It would be of great advantage if foreign gene expression under CaMV 35S control increased. The value of field trials in putting theory to practise is also evident from these results.

During the different *in vitro* manipulation and development stages which the transgenic tobacco plants passed through in this study, a level of developmental regulation of the CaMV 35S promoter could be detected. Samples of *in vitro* grown plants showed high levels of 35S-*uidA* activity when assayed for ß-glucuronidase (GUS) activity. In mature plants, however, the level of *uidA* gene activity declined. This level could be restored to *in vitro* levels by culturing mature transgenic leaf samples *in vitro* on tissue culture medium to induce organogenesis. Hensgens *et al.* (1992) studied the influence of growth conditions and plant age on the expression of different *gusA* (*uidA*) constructs. Despite high levels of *uidA* mRNA transcripts in older leaf and root material, the GUS activity was lower than than found in *in vitro* grown material (Hensgens *et al.*, 1992). A possible explanation for this discrepancy could be the possible post-translational control of the *uidA* gene (Kuhlmeier, 1992).

Another possible explanation for increased *uidA* activity *in vitro* is that the *in vitro* mode of growth is stressful to plants because of the confined nature of the culture environment under these conditions. *In vitro* stress is thought to be sufficient, for example, to promote somaclonal variation and mutation in plant material in culture (Bellini *et al.*, 1992). If stress increases CaMV 35S promoter activity, *uidA* gene

expression might increase under *in vitro* conditions. Mature hardened off plants do not experience this stress, but once samples are placed back in *in vitro* culture, stress occurs again.

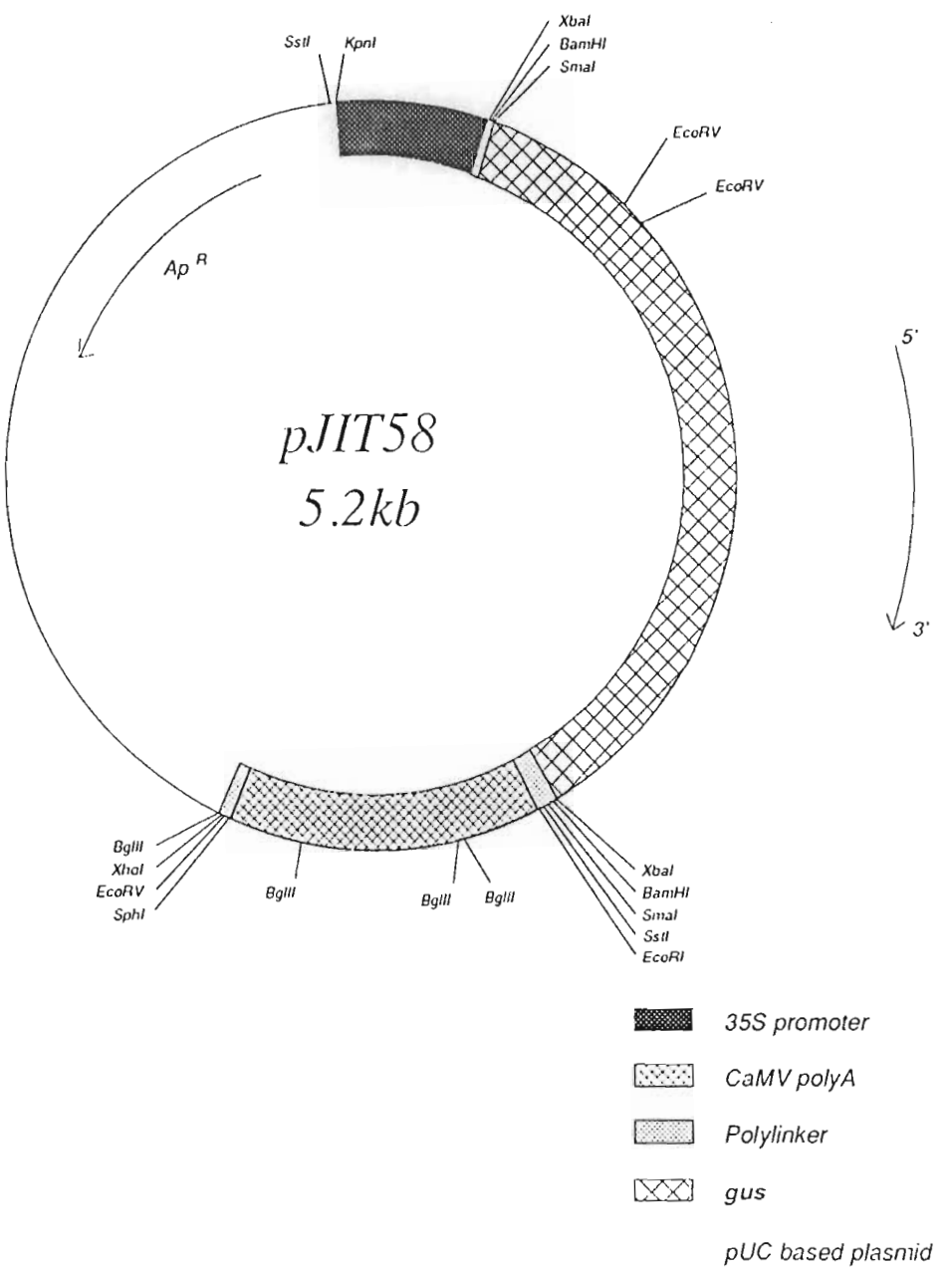
Observations made by Williamson *et al.*(1989) suggest that the CaMV 35S promoter is not constitutively active, but is merely restricted to actively dividing tissues in plants. Although the phloem cells are actively dividing within the plant, other cell types are also undergoing cell division during plant growth. However, the *uidA* gene expression does not track cell division within the plant, and is always focussed solely in phloem and glandular hair cells, in both vegetative and reproductive structures. Factors other than just cell division must play a role in the tissue specific activity of the CaMV 35S promoter. However, the role of cell division in CaMV 35S promoter activity would explain some of the observations made in this present study. When cells of mature transgenic tobacco leaves (low GUS activity) was induced to divide rapidly by culture *in vitro* on tissue culture medium to promote either adventitious shoot induction (differentiation) or callus formation (dedifferentiation), the 35S-GUS activity became stronger than in the original leaf pieces. Clearly, many factors are involved in CaMV 35S promoter regulation.

The features of the CaMV 35S promoter could be expected from the fact that the promoter is genetic machinery derived from a plant virus. The CaMV virus is aphid transmitted (Covey *et al.*, 1990). As aphids are usually phloem feeders, the optimal location of virus replication to assist the insect vector in transmitting the virus to new host plants would be the phloem cells. Virus particles are also rapidly spread throughout the plant through systemic movement through the vascular system. Dissemination of virus particles in young developing leaves occurs possibly either by cell-to-cell spread or by division of cells containing virus particles (Covey *et al.*, 1990). Some intense 35S-GUS activity was also associated in the glandular hairs and this has been reported on by other authors (Jefferson *et al.*, 1987). The glandular hairs of some plants function to attract insects to the plant (Wagner, 1991). If one assigns some purpose strategic to the CaMV virus in possessing a

promoter that functions in glandular hair cells, it is possible that the location of virus replication in these organs could also be part of the virus transmission strategy. Perhaps through sticky glandular hair secretions (containing virus particles) adhering to visiting insects, the CaMV virus could be mechanically transmitted. However, if the major site of virus replication and systemic translocation is the phloem cells of susceptible plants, it is difficult to imagine how virus or virus DNA becomes located in the glandular hair cells.

In conclusion, this study resulted in the production of transgenic tobacco lines containing a resistance gene for the herbicide asulam. The expression of the asulam resistance gene as well as accompanying marker genes in transgenic tobacco plants was fully investigated, as well as the inheritance of these genes by progeny. It also provided an opportunity to investigate and apply some of the molecular techniques which would be required to comply with South African and European legislation in order to prepare plants for transgenic field trial testing. Field trial testing is an essential part in steps towards commercializing transgenic crop plants. Further more, the transgenic tobacco plants which were produced were available for an investigation of some of the characteristics of the cauliflower mosaic virus 35S promoter, and to enable speculation on the adaptive value of these properties to the CaMV virus itself.

APPENDIX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XhoI
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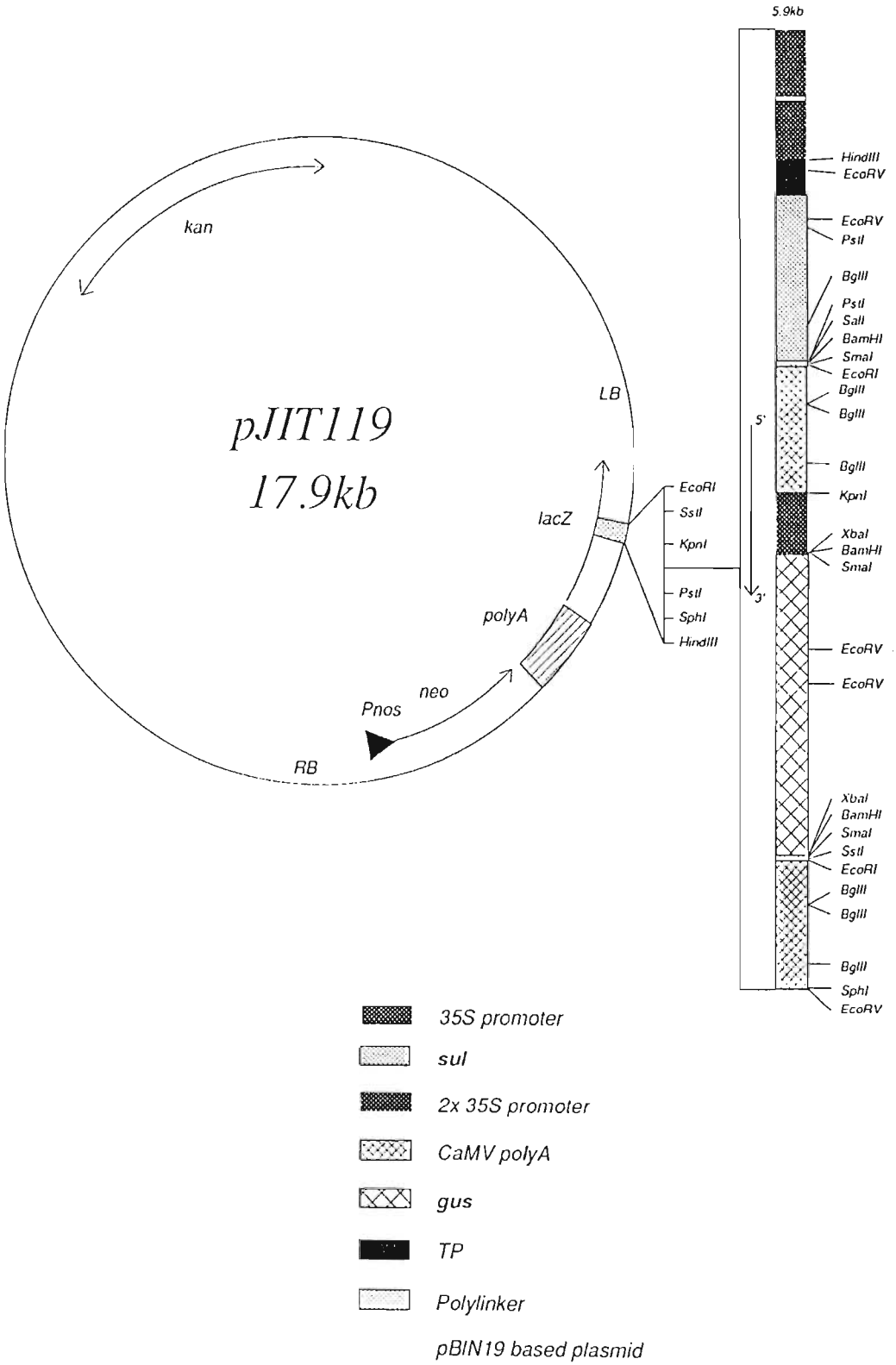
EcoRI
35S promoter
CaMV polyA
Polylinker
gus
pUC based plasmid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PstI
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SmaI
EcoRI
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KpnI
XbaI
BamHI
SmaI
EcoRV
EcoRV
XbaI
BamHI
SmaI
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BglII
BglII
SphI
EcoRV
5'
3'
kan
pJIT119
17.9kb
LB
lacZ
polyA
neo
Pnos
RB
EcoRI
SstI
KpnI
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2x 35S promoter
CaMV polyA
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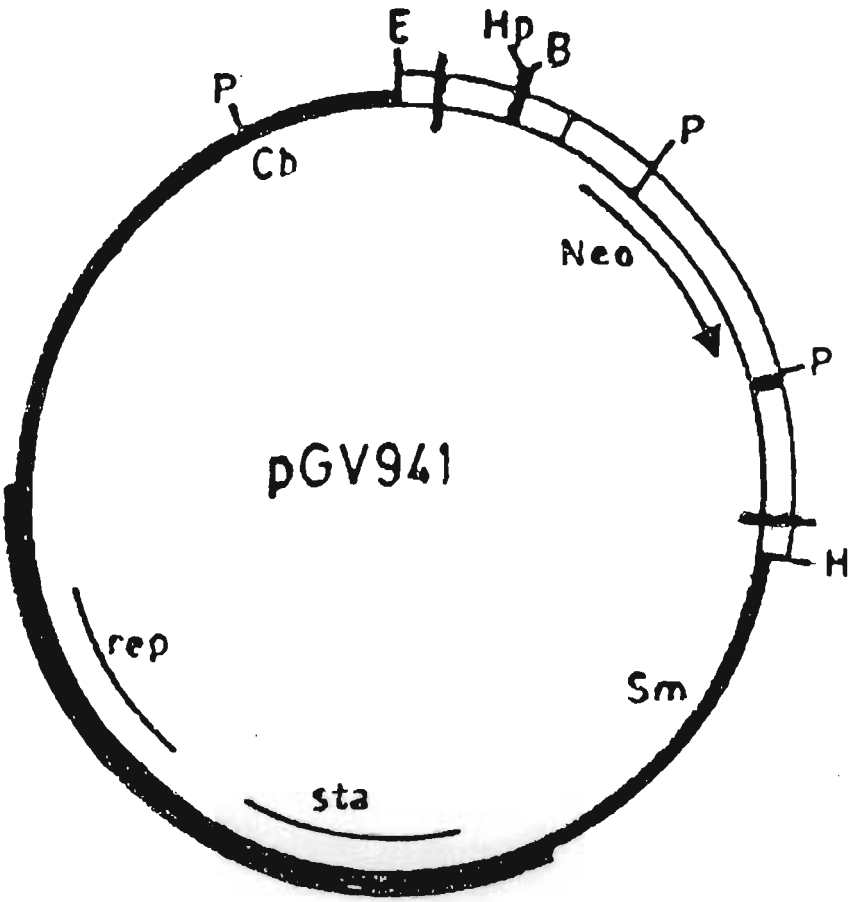
E
Hp
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