THE TRANSFORMATION OF SOUTH AFRICAN SOYA BEAN CULTIVARS WITH A SYNTHETIC BASTA RESISTANCE GENE

By

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Submitted in fulfilment of the requirements
for the degree of

MASTER OF SCIENCE IN AGRICULTURE

in the
Department of Genetics
Faculty of Agriculture
University of Natal
Pietermaritzburg
November 1995

ABSTRACT

The development of a genetic engineering system for soya bean (*Glycine max* L.) is described in this thesis. Routine tissue culture regeneration systems were developed for South African cultivars of soya bean despite the recalcitrant nature of this plant to *in vitro* manipulation. Regeneration of shoots was obtained when cotyledons were excised from seeds germinated for two days and cultured on B5 BA 20 medium containing 2 mg/l BA. The important problems of *in vitro* shoot elongation and rooting were overcome by culturing cotyledons in the dark for four weeks to produce shoots with unusually long stems. This was followed by one week of culture under conditions of high light intensity to obtain healthy green shoots which could be rooted, either in sorbarods or on solid ½MS 30 medium. The use of a mist bed for the hardening off of rooted soya bean regenerants was essential for the recovery of fertile soya bean plants. Molecular techniques for the cloning of foreign genes into binary vectors suitable for plant genetic engineering were also studied and are described in the thesis. The Basta herbicide resistance gene, *pat*, was successfully cloned into the binary vector pBI121 which contains the β-glucuronidase (GUS) reporter gene, *uid*A. The new construct, pBI121/Ac, was conjugated into various disarmed *Agrobacterium tumefaciens* strains and these strains, along with other binary vector-containing strains, were used to transform soya bean plant material. Although a protocol for the routine transformation of soya bean was not developed, transgenic soya bean material resistant to kanamycin and showing GUS activity was obtained. Transformation of wound sites on cotyledons was obtained in several experiments and transgenic shoots were regenerated from inoculated cotyledons. Only the *A. tumefaciens* strain C58C1(pGV2260)(pJIT119) was

able to transform cotyledonary cells of soya bean and, therefore, only kanamycin resistant soya bean shoots were produced. Transgenic soya bean plants resistant to the herbicide Basta were not produced due to the recalcitrant nature of the crop to genetic engineering. Transformation of the non-recalcitrant plant, tobacco, which is a model system for plant genetic engineering was achieved. The binary *pat* gene containing vector constructed in this study, as well as vectors obtained from AgrEvo, were tested. The transgenic Basta resistant tobacco plants obtained were used to optimize assay systems for the analysis of transformed plant material containing the *pat* gene. These assay systems included the use of the polymerase chain reaction as well as digoxigenin-labelling of a DNA probe suitable for detection of the *pat* gene.

DECLARATION

The experimental work described in this thesis was carried out part-time at Food Science and Technology, CSIR, Pretoria, from August 1991 to November 1995.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

It is requested that the findings contained in this thesis be kept confidential for a period of five years from the date of submission due to an ongoing contract relating to this work.

Tracy van Huyssteen

November 1995

ACKNOWLEDGEDGEMENTS

Sincere thanks are extended to:

Mr W Goodwin of AgrEvo SA (a subsidiary of Hoechst), without whom this project would never have been started.

AgrEvo SA and AgrEvo AG for funding this research.

Professor M Wallis, my supervisor, for his help, and in particular, for his valuable comments on the final draft of this thesis.

Dr J Webster, my co-supervisor for affording me the opportunity to carry out this research at the CSIR.

Dr S Hearn, my project leader at the CSIR for providing tremendous help, advice, encouragement and constructive criticism, and in particular, for always being willing and able to read and comment on numerous drafts of this thesis.

My friends and colleagues at Food Science and Technology, CSIR, for their advice and encouragement.

J Mathabe, R Khonothi, J Mabuse and J Malaka for technical assistance.

My parents for their unfailing support, encouragement, advice, financial assistance and everything else they have done for me over the years.

My mother for typing the bulk of this thesis.

My husband for his invaluable support and for his help with the typing of the tables and figures in this thesis.

CONTENTS

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy-acetic acid
ABA	abscisic acid
Ap^R	ampicillin resistance
AS	acetosyringone
BA	6-benzyl aminopurine
bp	base pair
Bp	bialaphos
°C	degrees centigrade
CaMV	cauliflower mosaic virus
CAT	chloramphenicol acetyltransferase
Cb^R	carbenicillin resistance
cm	centimetre
cv	cultivar
cvs	cultivars
Cx	cefotaxime
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetra-acetic acid
FAA	formyl-aceto-alcohol
g	gram
GA_3	gibberellic acid
Glu Am	glufosinate ammonium
Gm^R	gentamycin resistance
GS	glutamine synthetase
GUS	β-glucuronidase
Hm^R	hygromycin resistance
IAA	3-indole-acetic acid
IBA	3-indole-butyric acid
kb	kilobase
Km	kanamycin
Km^R	kanamycin resistance

l	litre
LA	Luria agar
LB	Luria broth
M	molar
Mag	magnification
$\mu E.m^{-2}.s^{-1}$	microeinsteins
µg	microgram
µl	microlitre
MIC	minimum inhibitory concentration
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
$m.s^{-1}$	metres per second
NAA	1-naphthyl-acetic acid
NBT	nitroblue tetrazolium salt
ng	nanogram
no	number
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picogram
PPT	phosphinothricin
$RecA^{-}$	recombination deficient
Rf^{R}	rifampicin resistance
rpm	revolutions per minute
sdH_2O	sterile distilled water
Sm^{R}	streptomycin resistance
Sp^{R}	spectinomycin resistance
TAE	tris-acetate
TBE	tris-borate
TE	tris-EDTA
Tc^{R}	tetracycline resistance
T-DNA	transferred DNA
Ti	tumour inducing
TVP	textured vegetable protein
UV	ultraviolet
X-glu	5-bromo-4-chloro-3-indolyl glucuronide
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate toluidinium salt

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The genetic manipulation of crop plants has been carried out by man for thousands of years. In early times this was by a process of random hybridizations and selection among wild species. During the early part of the twentieth century it was found that genetic traits segregate in an ordered and predictable fashion. These traits can be recombined in a directed way to generate progeny which possess novel genetic characteristics. This led to the development of more useful plant varieties with desirable traits. Knowledge of plant physiology and plant biochemistry has also helped in the development of superior plant varieties (Lindsey, 1992).

Further advances in crop breeding were due to a greater understanding of plants and plant genetics. The transmission of traits in plants is complex. Plants have three genomes which are located in the nucleus, the mitochondria and the plastids. Because the male gametophyte contributes insignificant quantities of cytoplasm to the zygote, genes which are encoded in the mitochondria and the plastids are inherited in a non-Mendelian manner through the female line. This type of inheritance complicates plant breeding (Lindsey, 1992).

An insight into the genetic consequences of continued inbreeding or outbreeding, as well as incompatibility mechanisms which determine whether two plants will cross-fertilize successfully, has also influenced the stategies used by plant breeders to produce new cultivars (Lindsey, 1992).

There are limitations to crop improvement using conventional breeding methods. For example, traditional breeding techniques allow only the transfer and recombination of

whole genomes in plants. This means that the introduction of one valuable gene into a plant may be accompanied by less valuable or even deleterious genes which are genetically linked to it. Also, many agronomically important traits such as plant height, fruit texture and fruit flavour are determined by multiple genes (polygenes) which each have a small but additive effect on phenotype and create difficulties for plant breeders. Perhaps the greatest limitation to crop improvement using classical plant breeding methods however, is the potential reduction in the size of the gene pool which is available to plant breeders. This reduction is largely due to the demand by farmers and consumers for a relatively small number of crop varieties with uniform yield, quality and processing characteristics. Also, sexual incompatibility barriers often limit the transfer of potentially useful genes from related wild species. By relying on a limited number of genotypes which have a limited set of characteristics, the ability to develop novel varieties decreases (US Congress Citation, 1991; Lindsey, 1992).

1.2 THE USE OF PLANT BIOTECHNOLOGY TO IMPROVE CROP PLANTS

From the above examples, it is clear that crop improvement using conventional breeding practices is limited and that a new series of techniques which augment classical approaches to the genetic modification of crop plants are becoming important. The latest in a series of modern technologies which have the potential to increase the productivity of world agriculture is plant biotechnology. Modern plant biotechnology can be defined as the selective alteration of genetic material of plants to produce useful products. This technology is being used to develop new plant varieties with new characteristics that cannot be obtained by sexual means. Plant biotechnology includes techniques such as protoplast fusion, somaclonal variation, embryo rescue and plant genetic engineering (Vasil, 1990; US Congress Citation, 1991).

Protoplasts are plant cells which have had their cell walls removed by enzymatic degradation. Protoplasts of the same or different plant species can be fused and grown into whole plants with new characteristics which cannot be produced by conventional breeding methods. Although many somatic hybrid plants have been obtained using

protoplast fusion, few of these are actually useful. Perhaps the best use of protoplast fusion will be in the production of cybrids which contain the nuclear and cytoplasmic genomes of one parent but only the cytoplasmic genome of the second parent. These cybrids could be useful in the transfer of cytoplasmic male sterility into plants. This is an important trait in plant breeding (Vasil, 1990; US Congress Citation, 1991).

Somaclonal variation is the genetic variation found in plant cell cultures resulting from either pre-existing variations in somatic plant cells or from mutations generated during the growth of plant cell cultures. Very little of the variation found in cell cultures is recovered in regenerated plants and almost all of it is useless or similar to the variation obtained after sexual crossing. Despite the many potential uses claimed for somaclonal variation, no significantly important new variety of any major crop species has been developed as a result of somaclonal variation (Vasil, 1990; US Congress Citation, 1991).

Agronomically useful hybrids even between closely related species are often unobtainable due to sexual incompatibility. In many cases, fertilization takes place but the developing embryos abort at various stages of development. The technique of embryo rescue which involves the excision and culture of young hybrid embryos from plants has proved very useful in growing such embryos to maturity and in obtaining hybrid plants (Vasil, 1990; US Congress Citation, 1991).

Plant genetic engineering is the alteration of the DNA of a plant cell so that the cell can produce more or different chemicals, or perform completely new functions (Haas, 1984) and is discussed in further detail below.

1.3 PLANT GENETIC ENGINEERING

Plant genetic engineering has great potential in agriculture. By the turn of the century, we will be using crop products which have been designed to market specification by the

addition, elimination or modification of genes (Haas, 1984; Vasil, 1990; US Congress Citation, 1991).

By 1983, new molecular techniques, including recombinant DNA technology, provided the necessary tools to allow the first successful transfer of foreign DNA into tobacco (Herrera-Estrella *et al.*, 1983) and later other members of the *Solanaceae* (Horsch *et al.*, 1985), initiating the era of plant genetic engineering. Between 1983 and 1988, there was a huge increase in the number of plant species which could be genetically engineered, primarily as a result of the development of *Agrobacterium*-based vectors. At the same time, advances in plant cell biology enabled the recovery of plants from single cell and protoplast cultures. These advances stimulated research in the area of direct DNA transfer culminating in the development of electroporation and polyethylene glycol-mediated transformation for the transfer of foreign DNA into single cells. Direct DNA transfer methods resulted in a dramatic increase in the range of plant species which could be engineered. Noticeably absent from this list of plants were the important legumes and cereals. Members of both these families proved to be extremely recalcitrant to regeneration and transformation due to limitations in their cell culture response and problems with effective DNA delivery methods. The development of particle bombardment-based technologies removed these constraints and it is now possible to engineer almost all important legumes and cereals (O'Brien, 1992; Bud, 1993; Christou, 1994).

The ability to insert foreign genes into plants using recombinant DNA methods provides plant breeders with new stategies for plant modification and improvement. Research and field testing have recently been dominated by plants which exhibit either herbicide resistance, insect resistance or viral resistance. Altering other plant traits such as those affecting plant tolerance to environmental stress, for example drought and salinity, or traits that add value such as altered ratios of oils, fatty acids and amino acids, is more complicated because many of these traits are multigenic and therefore more difficult to transfer. Some of these traits can be modified through traditional breeding programmes but plant genetic engineering can improve the efficiency of the alterations and extend

the range of possible modifications (Vasil, 1990; US Congress Citation, 1991; Lindsey, 1992).

A major drawback with plant genetic engineering is consumer acceptance. As this is a new technology, there are a number of ethical and social issues that have to be considered. These include the cost and the safety of genetically engineered plants, the impact of transgenic products on world agriculture and the question of environmental risk following the release of transgenic plants. These issues will have to be examined before the products of this technology are allowed to become commercialized. As there are genetically engineered crops destined for commercial release in their third or fourth years of field tests, these issues will have to be resolved soon (Stolp and Bunders, 1989; Rissler and Mellon, 1994).

The development of a successful plant genetic engineering system for a particular plant involves the development of tissue culture methods for the regeneration of fertile plants from *in vitro* cultured plant material. Foreign genes suitable for transfer into plants, and gene transfer systems that allow efficient delivery of these genes into plant cells are also required. Additionally, in a plant genetic engineering system, it must be possible to select transformed plant cells from untransformed ones so that only transgenic cells survive and produce plants (Lin *et al.*, 1987; von Wordragen and Dons, 1992).

1.4 PLANT TISSUE CULTURE TECHNIQUES

Plant tissue culture refers to the process whereby small pieces of living tissue (explants) are excised from a plant and grown aseptically *in vitro* for indefinite periods of time on nutrient medium. The tissue culture of plants has many applications. Plant tissue culture is used for the rapid vegetative propagation of plants and to remove diseases, particularly viruses, from plants. It is also used to maintain disease-free stocks of plants in germplasm collections. Another application of commercial importance is the industrial scale culture of plant cells from which valuable chemical products can be extracted e.g. horseradish peroxidase (George and Sherrington, 1984; Yamada *et al.*, 1987). Perhaps

the most important use for tissue culture is its role in bringing about genetic change *in vitro* via selection of desirable traits using mutations and somaclonal variation, or by the transfer of foreign genes into plant cells via plant genetic engineering techniques. A prerequisite for the production of genetically engineered plants is the ability to regenerate a complete plant from the altered cell and it must therefore be possible to manipulate the plant material *in vitro* (George and Sherrington, 1984; von Wordragen and Dons, 1992).

Plant regeneration through tissue culture can be accomplished by embryo culture. somatic embryogenesis or organogenesis. In embryo culture, embryos are excised from seeds or ovules and placed on nutrient medium until they have developed into seedlings. Somatic embryogenesis is the production of embryo-like structures from somatic cells. Somatic embryos are not physically attached to the tissue of origin but are independent bipolar structures which can germinate into plantlets. Organogenesis involves the formation and outgrowth of adventitious shoots from an explant or from callus derived from an explant. Organogenesis also includes the production of plantlets from axillary buds. The shoots derived from organogenesis are unipolar structures and are physically connected to the tissue of origin (George and Sherrington, 1984; Tisserat, 1985).

For the purpose of genetically engineering plants, tissue culture techniques are required to bring about the multiplication of organs, tissues or cells that are able to give rise to new intact transgenic plants. Regeneration methods can be either direct or indirect. Direct shoot regeneration is the induction of shoots on explants with no prior formation of callus. Indirect shoot regeneration from morphogenetic callus occurs when callus pieces are repeatedly subcultured and then transferred to a shoot-inducing medium. Direct embryogenesis is the induction of somatic embryos on explants without prior formation of callus while indirect embryogenesis from embryogenetic callus or suspension cultures occurs when the callus or the suspension cultures are transferred to a medium favouring embryo development (George and Sherrington, 1984).

Well-documented examples of plant regeneration facilitating plant genetic engineering include the leaf disc method used for non-recalcitrant plants such as tobacco and chicory (Horsch *et al.*, 1985; Vermeulen *et al.*, 1992), regeneration from cotyledons in plants such as tomato (Fillatti *et al.*, 1987) and regeneration from suspension cultures of plants such as maize (Vain *et al.*, 1993). Plantlets are able to regenerate from transformed single cells at the edges of leaf discs or cotyledons, or from transformed cells in the suspension cultures (Horsch *et al.*, 1985; Fillatti *et al.*, 1987; Vermeulen *et al.*, 1992; Vain *et al.*, 1993).

Explant selection plays an important role in successful plant regeneration. Explants can range in size from seedlings and organs to single cells and protoplasts. The kind of explant used is determined by the type and purpose of the tissue culture process and the plant species. Almost any part of a plant can be cultured *in vitro* but explants must be obtained from healthy plants for the best results. They can be excised from sterilized seeds or they can be removed from *in vivo* grown plants and then surface sterilized e.g. leaf, root or bulb material. Immature tissues and organs are usually more responsive *in vitro* than mature ones and are therefore preferred (George and Sherrington, 1984; Mantell *et al.*, 1985).

Disinfection of plant material is required in order to eradicate surface micro-organisms as any contamination *in vitro* inhibits plant growth. Household bleach which contains the sterilant sodium hypochlorite is commonly used to sterilize the plant material. Contamination can often be reduced by washing plant material under running water for several hours, by soaking in 70% ethanol for one or two minutes prior to treatment, by adding a wetting agent such as Tween to the sterilant, by reducing the air pressure in the container, by shaking the container or by adding antibiotics to the germination medium. Following sterilization, the plant material must be rinsed several times in sterile distilled water to remove any traces of the sterilant. It is important to optimize the length of time the plant material spends in the sterilant and the concentration of the sterilant so that micro-organisms are destroyed but no damage to the plant material occurs. These factors vary with the plant species, the cultivar and the explant. It is

important to note that internal contaminants may occur and that these cannot be eliminated by surface sterilization (George and Sherrington, 1984; Tisserat, 1985).

Explants can be cultured in a liquid medium or in a medium which has been solidified with gelling agents such as agar or gelrite. The components of media used for the growth of plant material can be classified into seven groups according to the way in which stock solutions are prepared and stored. These groups are: inorganic macro-nutrients; inorganic micro-nutrients; an iron source; a carbon source; vitamins; plant growth regulators; and optional organics such as casein hydrolysate (George and Sherrington, 1984; Dixon, 1985; Mantell *et al.*, 1985; Tisserat, 1985).

There are four major classes of growth regulators which are important in plant tissue culture, namely auxins, cytokinins, gibberellins and abscisic acid. Without these substances, no differentiation occurs *in vitro*. An auxin and a cytokinin are usually both added to the tissue culture medium to obtain morphogenesis. The ratio of auxin to cytokinin determines the type and extent of growth in plant cell cultures (Figure 1.1) and must be optimized for each type of explant from each type of plant (George and Sherrington, 1984; Torres, 1989).

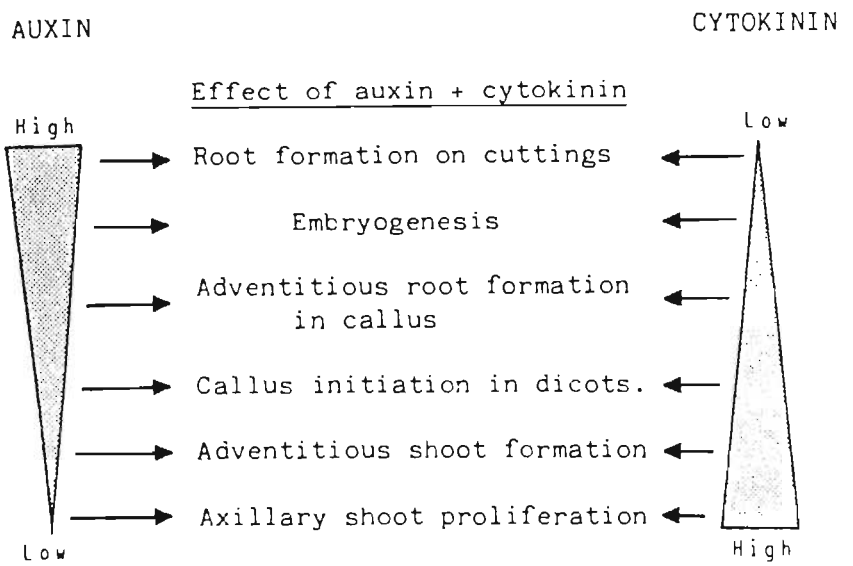

Figure 1.1 Relative amounts of auxin and cytokinin generally required to bring about *in vitro* plant morphogenesis (George and Sherrington, 1984)

The auxins commonly used in plant tissue culture media are IAA (3-indole-acetic acid), IBA (3-indole-butyric acid), 2,4-D (2,4-dichlorophenoxy-acetic acid) and NAA (1-naphthyl-acetic acid). They are usually added to a medium to stimulate callus production and cell growth, to initiate shoots and roots, to induce somatic embryogenesis and to stimulate growth from shoot apices and shoot tip cultures. The hormones BA (6-benzyl aminopurine), kinetin (6-furfuryl aminopurine) and zeatin (4-hydroxy-3-methyl-trans-2-butenyl aminopurine) are examples of cytokinins used in tissue culture. When added to a medium, they stimulate cell division, induce shoot formation and axillary shoot proliferation, and inhibit root formation (George and Sherrington, 1984; Torres, 1989).

Gibberellic acid (GA_3) and abscisic acid (ABA) are occasionally used in culture media. In most cases, cultures will grow without GA_3 or ABA but growth in some species can

be enhanced by their inclusion in the medium. Gibberellic acid promotes low density cell culture growth, enhances callus growth and elongates dwarfed or stunted plantlets. Abscisic acid inhibits or stimulates callus growth depending upon the plant species, enhances shoot or bud proliferation and inhibits latter stages of embryo development (George and Sherrington, 1984; Torres, 1989).

Generally, the macro-nutrients, the micro-nutrients and the iron source of one of the standard basal media such as B5 (Gamborg *et al.*, 1968) or MS (Murashige and Skoog, 1962) are used in tissue culture. The addition of the other media components, for example the carbon source, vitamins, plant growth regulators and optional organics are usually optimized for different plants and explants (George and Sherrington, 1984).

Apart from the media components, a variety of other factors influence the growth of plant material in culture and often interact with one another. These factors include the genotype of the plant (there are cultivar differences as well as species differences in the *in vitro* response of plants), the temperature at which cultures are kept (the average is 25°C), the humidity (the relative humidity must be around 70% or the cultures dry out rapidly), the light (wavelength, intensity and photoperiod must be optimized), the size of containers (linked to the build-up of carbon dioxide, ethylene and other volatiles). and the type of explant used (different explants respond differently *in vitro*) (George and Sherrington, 1984). Due to the large number of variables, it is obvious that optimizing a tissue culture system for a particular plant, its cultivars and even the diffent explants is a long-term project. This is especially difficult and time-consuming when plants are recalcitrant to regeneration as is the case with soya bean.

1.5 PLANT TRANSFORMATION TECHNIQUES

The development of genetic engineering methods for plants makes it possible, theoretically at least, to transfer any foreign gene into any plant species, overcoming some of the limitations of classical plant breeding methods. Bacteria and other plant

species are generally the source of heterologous genes for plant genetic engineering (Christou *et al.*, 1987; Chee *et al.*, 1989; Lindsey, 1992).

1.5.1 *Agrobacterium*-mediated gene transfer systems

The most widely employed transformation system for dicotyledonous plants is the vector-based system which makes use of the natural gene transfer properties of the soil-borne bacterial plant pathogen *Agrobacterium tumefaciens*. This bacterium, which induces crown gall disease in a number of dicotyledonous plant species, contains a large circular plasmid of about 200 kilobases (kb). It has been shown that this plasmid is responsible for the development of tumourous growth of infected plant tissue and is called the Ti (tumour-inducing) plasmid. The properties of the Ti plasmid have been exploited to develop Ti-plasmid based vectors for the transfer of new traits into plants (Lindsey, 1992).

Three regions of the *Agrobacterium* genetic material are now known to be involved in plant transformation activity. An 11 kb region of the *Agrobacterium* chromosome controls the attachment of the bacteria to the plant cells. The second region of plasmid origin is transferred into the plant cell and is approximately 20 kb in size. It possesses genes encoding enzymes involved in the biosynthesis of auxins, cytokinins and unusual amino acids called opines. The synthesis of auxins and cytokinins under bacterial transcriptional but plant translational control at a wound site on a plant results in the tumourous phenotype, while the opines, also synthesized in the plant cells, provide a source of nitrogen for the bacteria. The 20 kb region of plasmid DNA in which these genes are located is defined at its borders by 25 base pair (bp) inverted repeats. This region, excised from the Ti plasmid and integrated into the plant genome, is known as the T-DNA (transferred DNA). The excision events are in turn controlled by the third important region of the Ti plasmid, the 35 kb virulence (*vir*) region. It encodes a number of genes which are induced by phenolic signal molecules, such as acetosyringone, which are released by wounded plant cells (Lindsey, 1992).

When a wounded plant is exposed to a suspension of *Agrobacterium* cells at concentrations above 1 x 10^7 bacteria/ml, between 100 and 300 bacteria bind to each plant cell at the wound site . The *vir*A gene product is thought to recognize and interact with phenolic substances from the plant and transmit a signal to the bacterial cell. This signal activates the *vir*G gene product which in turn activates the other virulence genes (*vir*B, C, D and E) on the Ti plasmid. The 25 bp direct repeat sequences (borders) are recognized by a site specific endonuclease encoded by the *vir*D operon. Endonuclease activity produces single-stranded nicks within the border sequences and a single-stranded linear molecule (the T-DNA) is released from the Ti plasmid. It is thought that the *vir*E gene products may be involved in the transfer and integration of the T-DNA into the plant genome (Depicker *et al.*, 1985; Klee *et al.*, 1987; Armitage *et al.*, 1988; Lindsey, 1992). The interaction of *Agrobacterium* with a plant cell and the processes involved in T-DNA transfer are shown in Figure 1.2.

The development of *Agrobacterium* vector transformation systems to transfer novel genes into plants was based on several observations. The first was that the T-DNA genes that cause the tumours (oncogenes) are not required for T-DNA transfer or integration and that any DNA placed between the border sequences will be transferred to a plant. Secondly, the virulence genes (*vir*) of the Ti plasmid which code for products which allow T-DNA transfer to occur, can act in *cis* (when the T-DNA and the *vir* region are on the same plasmid) or in *trans* (when the T-DNA and the *vir* region are on separate plasmids). Thirdly, because only the border sequences of the T-DNA are required for plant transformation, vectors can be developed which contain these sequences but which have the rest of the T-DNA deleted or replaced by foreign genes. Transformation vectors based on T-DNA from Ti plasmids therefore do not need to contain oncogenes and plants transformed with these avirulent vectors can regenerate into normal fertile plants. Ti plasmids which lack the oncogenes are known as disarmed plasmids (Kleinhofs, 1985; Bryne *et al.*, 1987; Rogers *et al.*, 1987; Armitage *et al.*, 1988; Peleman and de Clercq, 1989).

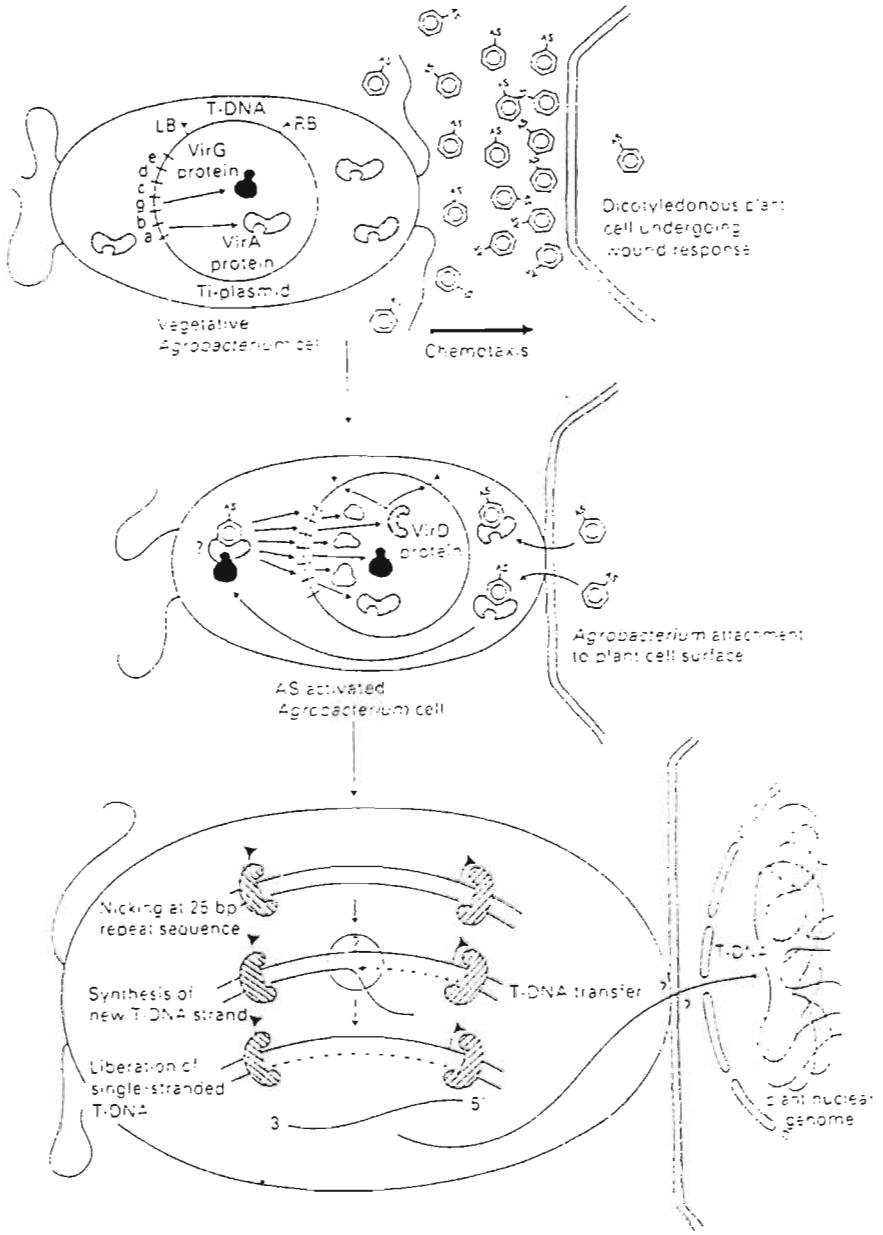

Figure 1.2 *Agrobacterium*/plant interaction and mechanism of T-DNA transfer (AS = acetosyringone) (Armitage *et al.*, 1988)

There are two types of non-oncogenic vectors, namely co-integrate (*cis*) vectors and binary (*trans*) vectors. Co-integrate vectors (Figure 1.3) are derived from a wild-type Ti plasmid in which the oncogenes are replaced by a segment of DNA that has a region homologous to a small cloning vector (intermediate vector) that only replicates in *Escherichia coli*. Foreign genes can be inserted into the intermediate vector in *E. coli* which is then transferred to *A. tumefaciens* by bacterial conjugation where a single crossover occurs between the homologous sequences in the Ti plasmid and the cloning vector to produce the co-integrate structure. Co-integrate vectors are useful because once they are integrated into the plasmid, they are stable. However, transformation frequencies are usually fairly low with this system and co-integrate vectors are now redundant (Armitage *et al.*, 1988; Peleman and de Clercq, 1989).

In the binary vector system (Figure 1.3) the T-DNA does not have to be physically joined to the Ti plasmid for it to be transferred into plant cells. The virulence functions remain intact on the Ti plasmid while the T-DNA is placed on a much smaller plasmid (binary vector) that can be manipulated easily *in vitro* and which replicates autonomously. Foreign DNA can be inserted into the binary vector using *E. coli* as a host. This construct can then be transferred to *A. tumefaciens* which contains a helper Ti plasmid (with *vir* region). Binary vectors must contain: origins of replication which function in both *E. coli* and *Agrobacterium*; unique cloning sites; plant and bacterial selectable markers; and the T-DNA borders which are required to transfer the T-DNA into the plant cells. Binary vectors do not require co-integration and the frequency of transformation is usually higher with this system than with co-integrate vectors. Only binary vectors are in common use at present (An, 1987; Deblaere *et al.*, 1987; Klee *et al.*, 1987; Rogers *et al.*, 1987; Peleman and de Clercq, 1989).

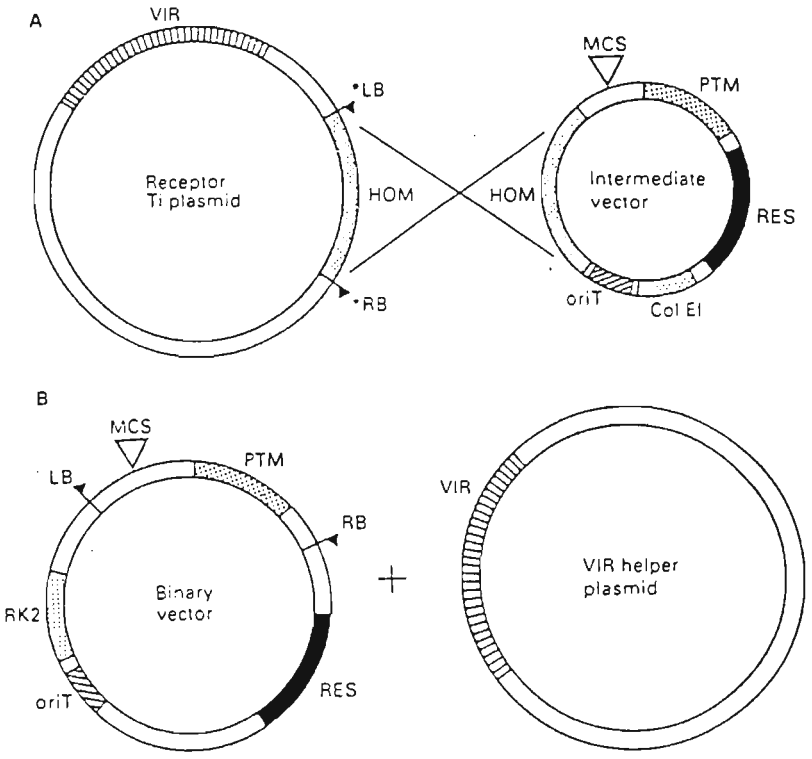

Figure 1.3 Schematic diagram of A) generalized co-integrate and B) generalized binary vector systems. VIR: virulence region; HOM: homologous regions within which recombination may occur for co-integration; LB,RB: left and right borders (*either LB or RB or both may be present on the intermediate vector); MCS: multicloning site; PTM: plant transformation marker; RES: antibiotic resistance marker to select for presence of vector sequences in bacterial hosts; oriT: origin of transfer and *bom* site for conjugative mobilization of vectors; ColE1: origin of replication from plasmid ColE1; RK2: wide host range origin of replication from plasmid pRK2 (Armitage *et al.*, 1988)

Most *Agrobacterium*-mediated transformation methods are based on the leaf disc transformation system of Horsch *et al.* (1985). Typically, an explant is inoculated with a strain of *A. tumefaciens* carrying transformation vectors. Co-cultivation is carried out for two days to allow induction of the *vir* genes and transfer of the T-DNA into the host genome. The explant is then transferred to a medium designed to induce shoot regeneration. This medium contains antibiotics to kill the *Agrobacterium* and selective agents which inhibit the regeneration of untransformed tissues. Regenerated shoots are in turn excised from the explant and transferred to a new medium (also containing antibiotics) for root induction (Lindsey, 1992).

At all stages after plant-bacterium co-cultivation, a selection pressure is maintained on the regenerating plant tissues so that only true transformants are generated. In practice, however, selection systems are rarely 100% efficient and some "escapes" may emerge. For this reason a detailed molecular analysis of putative transformants must be carried out to demonstrate the integration of foreign DNA sequences in the plant genome. The inclusion of screenable reporter genes in the transforming DNA, such as β-glucuronidase (GUS) (Jefferson *et al.*, 1987) or chloramphenicol acetyltransferase (CAT) (Töpfer *et al.*, 1988) can facilitate the analysis of regenerated plants (Lindsey, 1992).

The host range of *A. tumefaciens* is limited. While more than 20 crop species have now been transformed using this system, it has not proved useful for a number of recalcitrant dicotyledonous plants such as legumes (although the number of successes in this area is increasing). *Agrobacterium* has been found to be completely ineffective for the transformation of the commercially important cereal crops such as maize, wheat and barley although limited success has been reported in the case of rice. In response to the limitations in the application of *Agrobacterium*, efforts have been directed to the development of alternative vector-free or direct gene transfer systems for plant transformation (Lindsey, 1992).

1.5.2 Direct gene transfer systems

Direct gene transfer systems are characterized by the transfer of naked DNA molecules into, most commonly, protoplasts which are plant cells lacking cell walls. DNA transfer is followed by the regeneration of stably transformed callus and plants under antibiotic selection. Techniques for the introduction of DNA into protoplasts can be considered as falling into three classes, namely chemical and electrical methods, and the technique of microinjection. In all cases, the objective is to overcome the barrier of the plasma membrane so that DNA can move into the protoplast and into its nucleus (Lindsey, 1992).

Three chemical techniques have been used successfully to introduce DNA into plant protoplasts. These are calcium-DNA (Ca-DNA) precipitation, the use of liposomes and the use of polyethylene glycol (PEG). The basis of the Ca-DNA method is the formation of a co-precipitate of plasmid DNA with calcium phosphate, formed by adding $CaCl_2$ to a solution of DNA in a phosphate buffer. On contact with protoplasts, the co-precipitate is transferred across the plasma membrane in a calcium-requiring process. In order to encourage the endocytotic uptake of the precipitate, the protoplast/Ca-DNA complex is treated with polyvinyl alcohol and high pH calcium. This method was first used for plant transformation by Hain *et al.* (1985) using a tobacco leaf protoplast system. Transformation frequencies of 10^{-4} were obtained (Kuhlemeier *et al.*, 1987; Lindsey, 1992).

Liposomes are negatively-charged spheres of lipid into which the transforming DNA is encapsulated. The liposomes are allowed to fuse with protoplasts using substances such as PEG or glycerol as the fusogen. Also using tobacco leaf protoplasts, Deshayes *et al.* (1985) obtained transformation frequencies of 4×10^{-5} (Matthews, 1983; Kuhlemeier *et al.*, 1987; Lindsey, 1992).

The most commonly used chemical technique for introducing DNA into protoplasts employs PEG and was first described by Krens *et al.* (1982). Protoplasts are mixed with DNA and a solution of PEG, the latter at a final concentration of 13-20%. Uptake of the

DNA-PEG complex is induced by washing in a salt solution. Transformation frequencies of 10^{-4} to 10^{-3} were obtained for tobacco (Kuhlemeier *et al.*, 1987; Lindsey, 1992).

Increasingly popular and showing some spectacular results, is the use of electrical pulses to increase the permeability of the protoplast plasma membrane to DNA using a technique known as electroporation or electropermeabilization. It is perhaps the most widely used method of transforming protoplasts, and is potentially much simpler to use than chemical techniques. It is also the best understood in terms of its effect on plasma membrane permeabilization. Above a threshold field strength, electrical pulses produce irreversible damage to membrane structure, leading to cell death. Below this threshold value however, there may occur a non-lethal permeabilization termed "reversible dielectric breakdown", which is considered to be due to the transient formation or enlargement of pores in the lipid bilayer. The effects of electric fields on membrane permeability are relatively long-lived (minutes) compared with the duration of the applied pulse or pulses (micro- or milliseconds) and this allows time for DNA uptake to occur. Electroporation has been used successfully to transform protoplasts from which stable transformed callus of a number of crops, including maize, sugarcane and sugarbeet, has been generated. For many of these species, regeneration of whole transformed plants has proved difficult due to recalcitrance of the cultured protoplasts. Nevertheless, transformed plants have now been regenerated from eletroporated protoplasts of maize, although the plants were sterile, and oilseed rape, tomato, lettuce and rice for each of which fully fertile plants were obtained (Potrykus, 1989; Potrykus, 1991; Lindsey, 1992).

Another method used for the introduction of DNA into protoplasts is microinjection. Although this technique can also be applied to plant cells with cell walls, in practice it is very difficult to isolate single cells (liquid suspension cultures consist of cell aggregates) and to avoid the creation of chimaeric (non-clonal) colonies. Single protoplasts are therefore the usual target for injection. Because DNA can be injected directly into the nucleus using the microinjection technique, transformation frequencies by this route are relatively high compared to other methods of direct gene transfer. Furthermore, since the injected protoplasts are subsequently cultured in isolation, such as in microdroplets, the use of selection systems may be unnecessary. The major problem with this

approach is that it is very labour intensive and requires expensive equipment. It is possible to inject up to 100 nuclei per hour but a high protoplast plating efficiency is required to generate large numbers of transformants. The technique has not been adopted widely (Potrykus, 1989; Potrykus, 1991; Lindsey, 1992).

In no instances have chemical techniques been used successfully to introduce DNA into intact plant cells, and while there is some evidence that small levels of DNA uptake can be induced in plant cells by electroporation, no stable transformation of cells or plants has been reported via this method. Transgenic chimaeras of oilseed rape have been produced via microinjection of microspore-derived pro-embryos but this could not be repeated with other plant species. The intact cell wall therefore represents a major barrier to the movement of DNA (Potrykus, 1989; Lindsey, 1992).

Other methods of transferring DNA into intact cells rather than protoplasts have been studied. Methods include the use of a microlaser that can be used to burn holes into cell walls and membranes through which DNA can enter. Also tested was electrophoresis whereby DNA could be transported across cell walls. It is important to note, however, that none of these methods have proved useful for the routine genetic engineering of plants (Potrykus, 1989; Potrykus, 1991).

A method of direct gene transfer which is currently receiving much attention is the bombardment of cells or tissues with small metal microprojectiles which are coated with DNA. The microprojectiles which are beads (1-4 μm in diameter) of the dense metals gold or tungsten, have been shown to penetrate whole cells or organelles in a non-lethal fashion when accelerated at high velocities (up to 600 $m.s^{-1}$) by explosive discharge. The transferred DNA may either be expressed transiently in the absence of integration or may become stably incorporated into the genome of the cells of the bombarded explant. Transgenic plants of tobacco, soya bean, maize and many others have been produced using this technique. The advantage of the particle bombardment or biolistics method is that morphogenetic tissues such as immature embryos or apical meristems, rather than single cells or protoplasts, can be transformed. Successful regeneration of transformants following particle bombardment of morphogenetic tissues depends to a

large extent on the efficiency of the selection system, while the use of relatively large explants may overcome some of the tissue culture problems (regeneration) which often limit protoplast-based direct gene transfer systems. The particle bombardment method is the most commonly used direct DNA transfer method and by far the most successful (Carnes and Wright, 1988; Potrykus, 1989; Potrykus, 1991; Lindsey, 1992).

Agrobacterium and direct DNA transfer methods focus on the transfer of foreign DNA into the nucleus of the plant cell. Work is now beginning on plastid transformation for the expression of agronomically important traits. Proplastids of meristematic cells of flowering plants may differentiate into chloroplasts, amyloplasts or chromoplasts depending on the tissue type. Chloroplasts, the plastids found in photosynthetically active green cells have been most widely studied but the other plastid types have also received some attention. An advantage of plastid transformation is that, unlike nuclear transformation, there is no variation in foreign gene expression due to chromosomal position effects. This is because foreign genes are targeted to the plastid genome by homologous recombination. Also advantageous is that genes incorporated into the plastid genome are not pollen transmitted as plastids in most crop species are inherited exclusively from the maternal parent. The spread of foreign genes via pollen, which is a public safety concern, is therefore prevented. Plant cells contain up to 50 000 copies of the plastid genome so stable incorporation of a foreign gene into the plastid genome guarantees amplification of the foreign gene and can potentially result in a very high level of foreign gene expression. Plastid transformation is therefore likely to be studied in great detail in the near future (Carrer *et al.*, 1993; Maliga, 1993; Svab and Maliga, 1993; McBride *et al.*, 1994; Zoubenko *et al.*, 1994; McBride *et al.*, 1995)

1.6 SCREENABLE AND SELECTABLE MARKERS USED IN PLANT GENETIC ENGINEERING

Modification of the structure of the Ti plasmid of *A. tumefaciens* has allowed useful genes to be introduced into the T-DNA while the oncogenic genes, responsible for the expression of disease symptoms, have been removed. By physically separating the

virulence and T-DNA genes onto separate plasmids thereby creating binary vector systems, cloning genes into the T-DNA is greatly facilitated. Direct DNA plant transformation vectors are usually smaller and less complex than binary or co-integrate vectors used in *Agrobacterium*-mediated transformation systems but these direct DNA vectors have also been designed so that the cloning of foreign genes into the plasmid DNA is possible. This is mainly due to the presence of multiple cloning sites which facilitate gene cloning (Draper *et al.*, 1988; Lindsey, 1992).

A further modification of the vector plasmids needed for plant genetic engineering involves incorporating into *Agrobacterium* or direct DNA plant transformation vectors, one or more screenable and/or selectable marker genes. These genes are driven by promoters which are functional in plants, with the most commonly used promoter being the 35S transcript of the Cauliflower Mosaic Virus (CaMV) (Lindsey, 1992).

Examples of screenable markers are shown in Table 1.1. Screenable marker gene expression can be detected in single cells or localized in tissues by histochemical, fluorometric or colourimetric assays. The most commonly used screenable marker gene is the *uid*A gene coding for the enzyme β-glucuronidase (GUS) which cleaves glucuronide from any substrate it may be bound to. Various assays can then be used to detect enzyme activity. The most widely used detection method is the histochemical assay which produces a coloured precipitate at the site of enzyme activity. When β-glucuronidase acts on the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-glu), the indoxyl derivative produced undergoes an oxidative dimerization to form an insoluble, coloured dye, and plant and bacterial cells expressing the *uid*A gene turn blue (Herman, 1987; Jefferson, 1987).

Table 1.1 Examples of screenable markers used in plant genetic engineering (Lindsey, 1992)

Screenable Markers
Chloramphenicol acetyltransferase
β-glucuronidase
Nopaline synthase
Octopine synthase
Mannopine synthase
Agropine synthase
Luciferase
β-galactosidase

With regard to selectable marker genes, expression must result in products which allow the identification and selection of suitable dominant phenotypes. These markers include resistance to antibiotics and resistance to herbicides (Table 1.2). Agents that inhibit growth of non-transformed cells or slowly kill them are useful as selective markers. The neomycin phosphotransferase type II (*npt*II) gene, originally isolated from the prokaryotic transposon Tn5 has been most widely used as a selectable marker. The *npt*II gene product detoxifies aminoglycoside compounds such as kanamycin and G418 by phosphorylation. This marker however, cannot be used in some plants due to lack of sensitivity of the plants to aminoglycoside compounds or due to lack of selectability of the transformed cells (Kleinhofs, 1985; Klee *et al.*, 1987; Draper *et al.*, 1988).

Table 1.2 Examples of selectable markers used in plant genetic engineering (Lindsey, 1992)

Selectable markers
Neomycin phosphotransferase
Hygromycin phosphotransferase
Gentamycin-3-N-acetyltransferase
Dihydrofolate reductase
Phosphinothricin acetyltransferase
Streptomycin phosphotransferase
5-enolpyruvylshikimate-3-phosphate synthase
Acetolactase synthase
Bromoxynil nitrilase
Tryptophan monooxygenase
Indoleacetamide hydrolase
Isopentenyl transferase

1.7 GENETIC ENGINEERING OF RECALCITRANT CROPS

Despite the intense interest in the transformation of recalcitrant crops such as cotton (McCabe and Martinell. 1993), sunflower (Friedt, 1992), legumes and cereals (Christou 1994), only limited progress has been made. Few recalcitrant species can be routinely transformed and there is still little knowledge on the processes underlying transformation and regeneration of plant cells (von Wordragen and Dons, 1992; Christou, 1994; Hunold *et al.*, 1995).

Certain biological parameters affect the delivery of foreign genes into cells and the fate of these genes in the cells. It is known, for example, that not all plant cells are totipotent. Only certain plant cells or cell types in each tissue have transformation and/or regeneration abilities. Transgenic plants can only be regenerated from cells

competent both for integrative transformation and regeneration. It is important to note that only a very small percentage of cells in plant tissues are competent for both transformation and regeneration. Some cells are competent for either transformation or regeneration. Others are potentially competent i.e. they have the potential to shift to the competent state. The rest of the cells are non-competent and cannot be transformed or regenerated. The relative composition of cell populations in tissues is determined by the species, the genotype, the type of organ, the developmental state of the organ and tissue areas within the organ, and even by the individual history of the experimental plant. These differences result in plants such as legumes, cereals (Christou, 1994), chrysanthemum (von Wordragen and Dons, 1992), sunflower (Friedt, 1992) and cotton (McCabe and Martinell, 1993) being more recalcitrant to transformation and regeneration than, for example, tobacco and petunia (Lin *et al.*, 1987: Potrykus, 1991; von Wordragen and Dons, 1992).

Another factor which influences the ability of a plant species to be transformed and regenerated is the "wound response". The most effective trigger for shifting cells potentially competent for transformation into the competent state is wounding. Plant species differ in their wound response, as do different cells of the same plant. Only plants with a pronounced wound response develop large populations of wound-adjacent cells competent for transformation and regeneration. Plants that are recalcitrant to transformation with *Agrobacterium* probably do not express the appropriate wound response. This is most likely the reason for the failure of cereal plant transformation with *Agrobacterium*. It is not because they are monocotyledonous plants that cereals are difficult to transform but because they do not have the proper wound response. Monocotyledonous plants such as asparagus, which show wound response are as easy to transform as dicotyledonous plants capable of wound response. Dicotyledonous plants without proper wound response such as soya bean, pea, bean and peanut are as difficult to transform as cereals. Of note is the fact that wounding of cereal tissues does not lead to wound-adjacent cells competent for transformation and regeneration. Instead, wound-adjacent cells accumulate phenols and die. This means that it is unlikely that cereals will ever be able to be routinely transformed using *Agrobacterium*.

This may also be true for recalcitrant dicotyledonous plants (Potrykus, 1991; von Wordragen and Dons, 1992).

One of the main problems of *Agrobacterium*-mediated transformation of recalcitrant crops appears to be directing gene transfer towards those plant cells that are amenable to regeneration. With recalcitrant crops it is often more efficient to adapt the regeneration protocol to the transformation procedure as the transformation is usually more difficult (Potrykus, 1991; von Wordragen and Dons, 1992).

Few general rules apply to the development of *Agrobacterium*-mediated transformation protocols for recalcitrant crops. However, some aspects of transformation can be studied individually to improve the process. The main focus areas include the effect of the bacterial strain and the plant genotype on transformation. Some *Agrobacterium* strains are more virulent on some plants than on others due to the chromosomal background of the *Agrobacterium* used, the efficiency of the particular *vir* genes in T-DNA transfer, the function of the Ti plasmid regions outside the *vir* region and the T-DNA regions which enhance transfer. There are large differences in virulence among *Agrobacterium* strains but usually three strains corresponding to octopine, nopaline and succinamopine-agropine types are sufficient for screening for virulence on a particular host plant. The effect of host genotype may be due in part to varying responses to stress. The stress reaction of plants involves the secretion of phenolic compounds which direct the bacterium towards the wounded plant cells and switch on the *vir* genes. It is therefore important to test more than one plant genotype or cultivar. It is also important to choose plant genotypes that regenerate easily (Klee *et al.*, 1987; Rogers *et al.*, 1987; Armitage *et al.*, 1988; von Wordragen and Dons, 1992).

Another factor which affects the efficiency of gene transfer into a plant is the type and the age of the explants. In most cases, explants with actively dividing cells are the most susceptible starting material. The most successful *Agrobacterium*-mediated transformations are often found with embryos and shoot apices which have pre-existing meristems, or with cell suspensions, hypocotyls and cotyledons which have actively

dividing cells. Leaf explants are usually poor starting material for transformation of recalcitrant crops due to the lack of rapid cell division (von Wordragen and Dons, 1992).

Methods for enhancing transformation efficiency are often based on the observation that the *vir* genes are activated by phenolic compounds secreted by wounded plant cells. The most commonly used techniques are the culture of explants on feeder layers and the addition of phenolic compounds, usually acetosyringone, to the tissue culture medium. Feeder or nurse layers are suspensions of cells of a readily transformable plant species such as tobacco. Explants of recalcitrant plants are usually placed on this layer, separated from it by filter paper, one or more days prior to infection and throughout the co-cultivation period. The beneficial effect on transformation is probably due to the secretion of phenolic compounds by the nurse cells but other secreted compounds may influence dedifferentiation and regeneration of explant cells or diminish the negative effects of wounding and infection on the plant cells. Addition of acetosyringone to the tissue culture medium is generally only useful if the plants do not produce any of the phenolic compounds recognized by the bacterium or if the co-cultivation period is too short to allow induction, chemotaxis, attachment and gene transfer (von Wordragen and Dons, 1992).

Various components in the tissue culture medium, as well as environmental factors are known to affect the expression of the *vir* genes. For example, sucrose in the culture medium, an acidic pH and culture below 28°C are essential for the expression of the *vir*D and *vir*G genes. Other factors that influence transformation efficiency are those that reduce the effects of stress on plants. For example, $AgNo_3$ inhibits the ethylene response which triggers many stress reactions and has been found to be essential in transformation of *Brassica napus, B. oleracea* and less responsive potato genotypes. A preculture period prior to infection is often effective in reducing wounding stress. Infection stress can be reduced by optimizing the incubation and co-cultivation time and the concentration of transforming bacteria. Also, the method of selection can cause stress in the plant. Selection agents can inhibit regeneration of transformed material as the growth of transformed cells can be inhibited by the death of surrounding untransformed cells. To minimize this inhibition, the addition of selection agents to the

medium can be delayed. This allows plant cells to recover from bacterial infection before selection is applied (von Wordragen and Dons, 1992).

Some general guidelines which can be followed for the development of transformation protocols for recalcitrant crops have been outlined above. However, the optimization of these protocols remains largely empirical. Existing protocols are of limited use in the transformation of recalcitrant crops but can provide a better understanding of the processes involved. Certain dicotyledonous as well as monocotyledonous plants have proven to be recalcitrant and the development of successful and efficient transformation protocols for these species often requires several years (von Wordragen and Dons, 1992).

1.8 GENETIC ENGINEERING OF SOYA BEAN

Soya bean is an extremely recalcitrant crop with regard to *in vitro* transformation and regeneration. The genetic improvement of soya bean via molecular methods has therefore been limited due to the lack of an efficient transformation system and the inability to regenerate plants from transformed cells or tissues (Christou *et al.*, 1990). Detailed information on the regeneration and transformation of soya bean is given in the introductions to Chapters 2 and 4.

1.9 THE IMPORTANCE OF LEGUMES IN AGRICULTURE

Leguminous plants are found throughout the world. With more than 600 genera and 18 000 species, *Leguminosae* is the third largest family of flowering plants after *Compositae* and *Orchidaceae*. Its species are found in many diverse geographic and climatic areas, including temperate zones, humid tropics, arid zones, highlands, savannas and lowlands. Of all the crop plants used by man, only cereals are more important than legumes. However, while enormous resources have been expended in past years on research into modifying cereals such as rice, wheat, barley and maize,

only soya beans and peanuts among the legumes have received much attention in terms of genetic engineering (Christou, 1994).

Most crop legumes are very important ecologically and agriculturally because they have the ability to fix atmospheric nitrogen as a result of symbiotic relationships with soil microorganisms such as *Rhizobium*. As a result of their nitrogen-fixing ability and their high nutritive value for grazing, legumes are commonly used in crop rotation programmes (Christou, 1994).

Legume seeds, also known as beans, grain legumes or pulses, are richer in protein than cereal grains. Some, like soya beans and peanuts are also rich in oil. Most of the crop legumes are extremely valuable and nutritious due to the high levels of vitamins and protein in growing seedlings e.g. alfalfa and clover, and in seeds e.g. peas and beans. The seeds of legumes and cereal grains provide humans with approximately 70% of their dietary protein requirements. In developing countries, increased cultivation of legumes is the best hope for combating projected shortages in food supplies, especially in vegetable protein. However, some of the most important storage proteins in legume seeds are deficient in specific essential amino acids that must be made up in other ways. Researchers are trying to alter the amino acid composition of soya bean to correct this problem (Christou, 1994).

Of the thousands of known legume species, only a handful are used extensively today as human and animal food or for a variety of industrial products. These include soya bean, peanut, bean, pea, lentil, pigeon pea, chick-pea, mung bean, kidney bean, cowpea, alfalfa, clover and vetch. Because of its dual uses as a protein and oilseed crop, as well as the extensive acreage devoted to its cultivation, soya bean has been the primary target for genetic engineering in the *Leguminosae* (Christou, 1994).

1.10 THE IMPORTANCE OF SOYA BEAN AS A CROP

Soya bean (*Glycine max*) is one of the oldest crops grown by man. The plant was first grown in Eastern Asia and was cultivated for human consumption about 5 000 years ago. Soya bean was one of the most important crops in ancient China and Japan and was regarded as one of the five sacred grains required to sustain life. It was often referred to as "meat that grows on trees". Methods of preparation were many and varied and provided the people with meat, milk, cheese, bread and oil. The crop was introduced into Europe at the beginning of the 17th century but was not cultivated extensively until the 1900's when it became used primarily as forage. Soya beans were first processed into meal and oil in 1911. Up until 1935, most meal was used as fertilizer. Thereafter, the meal became widely used as livestock and poultry feed. By 1941, soya beans were grown mainly for the production of seed for processing (Harper, 1983; Women for Peace, 1984)

The United States presently supplies about two-thirds of the world's soya beans. They grow more soya beans than any other cultivated crop except maize and wheat, and export about 40% of the crop. Commercial varieties account for 98% of the soya bean grown in the United states and most of this seed is processed into soya bean meal and oil. Vegetable-type soya beans are used as whole beans or to produce bean sprouts. These seeds are usually green and are larger than those of commercial soya beans. The crop is a rich and inexpensive source of vegetable protein. The seeds contain 35 to 40% crude protein (compared to 18% for beef and fish) and approximately 30% carbohydrates, 18% fat, 10% water and 5% ash. The protein contains the eight essential amino acids in proportions which allow the protein to be readily taken up by the body. Apart from being a source of food for animals and humans, soya beans also supply raw materials for industry (Harper, 1983; Women for Peace, 1984).

Soya beans are used chiefly in the form of meal and oil worldwide. The seeds supply about a quarter of the world's fats and oils and two-thirds of the protein meal. The meal and the oil are produced by a solvent extraction process following mechanical cleaning and dehulling. Rollers are used to crush seeds into flakes. Crude oil is then extracted

from the flakes using a solvent. Once the oil has been removed, the flakes are referred to as soya bean meal. In the United States, over 95% of the crude flakes are heated and manufactured into high-protein feed for cattle, pigs, poultry and pets. The remaining meal is used as an ingredient in foods for human consumption. It can be finely ground into soya flour or coarsely ground into soya grits. The flour is used in baby food, cereals and some low-calorie products. Grits are used in sweets and processed meats such as hamburger patties and sausages. Baked goods and pet food contain both soya flour and grits (Widholm, 1988; Harper, 1983).

New food products were developed in the 1960's and 1970's by processing soya flour. When one sixth of the non-protein content is extracted from soya flour a product known as soya protein concentrate is produced. The concentrate is a cream that can be made into a powder or a grainy substance and is used in baby foods, cereals and processed meats. Isolated soya protein is produced when a quarter of the non-protein content is extracted from the flour. It provides firmness and protein in various processed foods. particularly meats (Harper, 1983).

A group of foods known as textured vegetable protein (TVP) are foods which are made up of extruded and spun soya protein. They are chemically treated to look and taste like meat so that they appeal to consumers. These TVP foods can be mixed with meat or eaten alone. They are cheaper than meat and contain more protein. Extruded soya protein is produced by mechanically shaping the soya flour into small meat-like pieces. The product may be dried before being packaged for sale. It is usually mixed with ground meat and becomes moist and chewy on the addition of water. Spun soya protein is made by spinning isolated soya protein into fibres. These products resemble meats such as beef, chicken and ham and are sold in canned, dried or frozen form (Harper, 1983).

Many foods contain soya meal but do not consist entirely of treated soya beans. These are known as soya derivatives and include food flavourings, soya sauce and soya milk. Soya milk does not contain lactose and is therefore easily digestible and as such, is excellent for babies. It has also been found that 62% of black South Africans are

lactose intolerant and a milk product which is lactose-free could be the answer to the problems experienced by feeding schemes throughout this country. Yoghurts, cream cheese and amazi (natural medicine food for healing malnutrition and diahorrea) can also be made from soya milk. Soya meal is also used in the manufacture of fertilizers, fire extinguisher fluid, insect sprays and paint (Harper, 1983; Women for Peace, 1984; Mkhuma, 1992).

Crude soya bean oil is made into three basic products namely, technical refined oil, edible refined oil and lecithin. Crude oil is purified by adding a mixture of water and alkali. The oil is then washed, dried, bleached by absorbent clay and passed through a filter to produce technical refined oil. This oil is used in the manufacture of candles, disinfectants, linoleum, soaps and varnishes. Edible refined oil is produced by deodorizing technical refined oil. This is accomplished by heating and steaming technical refined oil to remove its unpleasant odour and flavour (Harper, 1983).

Over 90% of the crude soya bean oil used in the United States is processed into edible refined oil. Half of this is used to make margarine and vegetable shortening. The rest is used in the production of cooking oils, mayonnaise, salad dressings and other food products. Edible refined oil is also found in adhesive tape, carbon paper, various drugs and explosives, and leather softeners. Lecithin is a sticky substance which is extracted from crude soya oil by mixing the oil with water. It is used in the manufacture of sweets, ice cream, baking products, chemicals, cosmetics and textiles (Harper, 1983).

The Western world has been slow to accept soya beans as a food source because of the large amount of time required to soften the bean through cooking and the characteristic unpleasant flavour that can develop in the cooked bean when an enzyme reacts with fats in the bean when it comes into contact with cold water. If not adequately heat treated, the trypsin inhibitor renders protein unusable by animals and humans but this trait has now been bred out of most soya bean cultivars. As with all beans, flatulence can be a problem. Also, the processed TVP has an unpleasant taste and a rubber-like texture. Soya bean was first introduced as a protein food in South Africa in the 1960's but was not well received because of the problems of the distinct

flavour, smell and texture. Presently however, many products have been developed which are acceptable to the Western palate making soya bean one of the world's most important agronomic crops (Harper, 1983; Women for Peace, 1984).

Soya beans are invaluable in easing the world food shortage and preventing malnutrition as the seeds contain more fats, proteins, vitamins, carbohydrates and minerals than most foods. Soya beans grown on 0.4 hectares of land can provide about ten times as much protein as beef cattle on the same land. Soya beans provide more protein than most other vegetables and grains and the growing of soya is therefore one of the most efficient uses of land (Guy, 1992).

1.11 SOYA BEAN CULTIVATION IN SOUTH AFRICA

At present soya bean is one of the most underestimated crops in South African agriculture. Apart from the benefit of the direct cash income from soya bean production, there are several other advantages to growing soya bean. These include the fact that soya bean has a beneficial effect on the soil structure, and in rotation with maize or winter wheat. complementary use is made of fertilizer since the nutrient requirements and root depth of the two crops differ. Soya bean fertilization is cheap because no nitrogen fertilization is required if root nodulation has occurred. Soya bean reacts to soil fertility rather than to direct fertilization. The preceding crop can therefore be thoroughly fertilized and any overfertilization will not be wasted. Soya bean is often rotated with maize, sorghum, millet or rice to enrich the soil with nitrogen. Soya bean production is cheaper than maize production. Where short and long-season crops such as soya beans and maize are grown together, better utilization of labour, implements and weed, insect and disease control occurs than in the case of monoculture (Harper, 1983; Smit, 1987; Smit and de Beer, 1991).

Soya bean is regarded as a catch crop or an emerging crop because it can still be planted after the last planting date for maize. Because of its photoperiodic sensitivity, the length of the soya bean growing period is proportionally decreased according to the

delay in planting date from the normal. This means that a considerable delay of the planting date will probably only cause a slight delay of the harvesting date. This contributes to soya bean's suitability as an emergency crop and can be fruitfully exploited. These factors, among others, make soya bean cultivation in South Africa worthwhile (Smit, 1987).

The soya bean plant is an upright annual legume with a wide morphological diversity. Plant height usually varies between 50 and 200 cm and the growing period between 70 and 180 days. There can also be great variation in growth behaviour of the same cultivar, depending on climate, environmental conditions and planting date. Usually soya beans are planted in the spring. After six to eight weeks, small flowers are produced. After a further two weeks, pods containing two to four oval to nearly round seeds (or beans) are borne over the entire length of the main stem and lateral branches. These develop for 30 to 40 days and mature as the leaves of the soya bean plant turn yellow and drop off. Soya bean is a self-pollinating crop that has a high probability of self-fertilization even before the flower opens. A distinction is made between cultivars with a determinate growth habit where vegetative growth ceases during the flowering stage, and those with an indeterminate growth habit where the plant continues growing after flowering. The flowers are either purple or white and the seed of commercially grown cultivars is cream-coloured with a colourless, brown or black hilum (Harper, 1983; Smit, 1987; Smit and de Beer, 1991).

In South Africa, soya beans are used predominantly as animal feeds for chickens and pigs. The proportion of soya beans used locally for direct human consumption is very small. Approximately 60 000 hectares annually are used for soya bean cultivation in South Africa. Between 80 000 and 120 000 tons of grain are produced per annum. The local requirement for soya beans far exceeds this but the main factor that has prevented increased local production has been price, in particular, the soya bean/maize price ratio. When the price ratio exceeds 2.5, soya bean production is favoured. With the poor maize price that was announced in 1994, a renewed interest in soya bean production was shown. Because of this, it is expected that soya bean production in South Africa will double within the next three years (A. Jarvie, Personal Communication).

1.12 AGRICULTURAL PROBLEMS ASSOCIATED WITH SOYA BEAN CULTIVATION IN SOUTH AFRICA

Worldwide, approximately 100 diseases and pests are associated with soya beans. Because the crop was, until recently, only grown on a limited scale in South Africa, there are relatively few pests that adversely affect soya bean cultivation. Viral diseases do occur, particularly soya bean mosaic virus which can lead to losses where conditions are favourable for the virus. The disease is transmitted through infected seed, by aphids and by mechanical actions and implement traffic on the land. It can be recognized by characteristic symptoms on both the seed and the plant itself. Virus-infected seed usually displays a spot on either side of and continuous with the hilum and the same colour as the hilum. Virus-infected plants have severely creased or wrinkled leaves and a mosaic pattern on the leaves when held up to light. There are less pods than normal and they are smaller and shinier than usual. In the field, virus-infected plants tend to remain green longer than virus-free plants. Control can be effected by planting virus-free seed, limiting aphid populations and uprooting infected plants (Harper, 1983; Smit, 1987; Smit and de Beer, 1991).

Bacterial diseases affect soya beans most severely during wet years. Symptoms occur mainly on the leaves of plants and vary from small yellow spots to large yellow spots to brown spots with yellow edges. A common bacterial disease is bacterial blight (*Pseudomonas glycinea*) which occurs particularly in cool, rainy weather. Large, irregular necrotic spots with watery, yellow margins appear on leaf tissue. The bacteria are transmitted via the seed and via plant residues. There is some doubt about the effect of bacterial blight on seed yield and there is no known practical control. Bacterial pustule (*Xanthomonas phaseoli*) occurs in hot areas with high rainfall. Lesions are found on the underside of leaves with raised brown pustules in the centre of the lesions. In regions where the disease already occurs, control can be effected by planting resistant cultivars (Harper, 1983; Smit, 1987).

Several fungal diseases have been observed in South African soya bean cultivars. Damage can vary from slight to severe. Seedlings, roots, stems, pods and seeds can be affected by fungal diseases. Downy mildew (*Peronospora manshurica*) can occur when temperatures are between 20 and 22°C. A white to brown down covers the undersides of leaves. In severe cases, fungicides can be used to control the disease. In areas where the fungus has built up in the soil, resistant cultivars can be planted. Sclerotinia stem rot (*Sclerotinia sclerotiorum*) is favoured by protracted wet periods and can cause severe damage. The disease is not common in soya beans in South Africa or the USA but if land is heavily infested, susceptible crops such as soya bean and sunflower should not be planted for a few years. Charcoal rot (*Macrophomina phaseolina*) occurs at the end of a hot, dry growing season and can be controlled by irrigation and rotational cropping. Purple spot (*Cercospora kikuchii*) is common in coastal areas where humidity is high during the seed-filling stage. It can be controlled by the use of fungicides or by planting resistant cultivars. Brown stem rot (*Phialophora gregata*) survives in soil on plant residues and where a build up in the soil occurs, non-host crops such as maize should be planted for at least three years (Harper, 1983; Smit, 1987; Smit and de Beer, 1991).

Moderate defoliation by leaf-eating insects usually has little effect on soya bean yields because of the large mass of leaves produced. However, where insects attack the pods, yields are directly affected and immediate action must be taken. Common soya bean pests are the bean leaf beetle, the green stinkbug, the velvet bean caterpillar and the American bollworm. These can be controlled by insecticides and by the removal of dead plant material in which these insects lay their eggs. Some soya bean cultivars that are resistant to insects have been developed. In the past, soya bean production in South Africa has been restricted to the heavier soils, mainly because of the crop's sensitivity to nematode infestation. However, there are now several cultivars available that are resistant to root-knot nematode (the major problem) and producers can now successfully grow soya beans on lighter soils. Although there are many soya bean diseases and pests, they are not a major threat at an economic level (Harper, 1983; Smit, 1987; Rosier, 1988).

A major problem in soya bean cultivation is the control of weeds. The first six to eight weeks following emergence are critical as young soya bean seedlings cannot compete with fast growing weeds and the potential yields are therefore reduced. The most important weeds of soya worldwide are grasses and sedges because they are very difficult to control. Many of the weeds can regenerate from small rhizome fragments and have sharp hairs which prevent hand weeding. Weed control in soya is usually accomplished using a combination of agronomic and chemical methods. The increased use of direct-drilling and minimum tillage in soya bean cultivation has led to increased weed problems and a subsequent increase in herbicide use. In the 1987/1988 crop year, soya bean herbicides accounted for 15% of the world's consumption of herbicides and 89% of the total world expenditure on agrochemicals for soya beans was spent on herbicides. In South Africa, in the 1989/1990 season, the figure was 33% and in the 1990/1991 crop year, 47%. This is expected to increase as more farmers realize that the use of herbicides increases yield (Rosier, 1988; Byford-Jones, 1992).

1.13 WEED CONTROL AND THE USE OF HERBICIDES

Although the use of herbicide resistance as a selectable marker for use in plant genetic engineering is becoming popular, the main reason for using herbicides is for the control of weeds in agriculture. Herbicides must be chosen so that most of the weeds are controlled at low levels of the herbicides. Broad-spectrum herbicides are generally more useful than narrow-spectrum ones. The inherent tolerance of the crop species to a particular herbicide must be studied before that herbicide is used to ensure that the herbicide is effective. To be useful, a herbicide must be selective i.e. it must allow uninhibited crop growth but must kill any weeds associated with the crop. Selectivity may be due to differences between the crop and the weeds in uptake, translocation, inactivation or breakdown of the herbicide. The ability of a herbicide to differentiate between a crop and a weed species determines its effectiveness and thus also limits the use of the herbicide (Pinthus, 1972; Faulkner, 1982; Chu, 1983).

Some weeds are difficult or expensive to control and the use of the herbicides themselves results in changes in the weed populations. These changes involve increases in the numbers of inherently tolerant weed species and increases in the evolution of tolerant species that were previously susceptible. These problems can be overcome by the development of new herbicides but this is time-consuming and costly and is becoming more difficult due to new chemical regulations and toxicology standards. Another problem is the increasing concern for the environment so the development of new cultivars which are tolerant to a particular herbicide (especially environmentally safe ones) is of interest (Faulkner, 1982; Chu, 1983).

Herbicide resistance in plants and microorganisms is encoded by genes and it is therefore possible to introduce this characteristic into plants either by conventional breeding methods or by employing molecular techniques. Existing herbicides can therefore be more widely used and fewer herbicides have to be developed and licensed if plants are genetically engineered to be resistant to existing herbicides. Herbicides can be used to control weeds associated with crops that were previously susceptible to the herbicide or selectivity of an existing herbicide can be improved. The development of herbicide-tolerant crops also allows the use of herbicides in crop rotations where one crop is susceptible to herbicide residues in the soil (Chaleff, 1981; Faulkner, 1982; Chu, 1983).

It is far easier to register an existing herbicide for use on a broader range of crops than it is to develop and register a new herbicide that is specific for a particular crop. It is cheaper and faster to utilize genetic means to breed a new crop variety that is resistant to a registered herbicide than it is to develop an entirely new herbicide. These crops, however, need to be as good as existing cultivars with regard to other characteristics unless the weed problem is so severe that poorer quality crops are still a viable alternative (Faulkner, 1982).

Single gene traits such as herbicide resistance are considered to be very attractive to agrochemical and seed companies for genetic engineering purposes. This is because it is much easier to engineer plants containing one foreign gene than it is to engineer

plants containing multiple foreign genes as is the case with insect and disease resistance. Many companies use herbicides in conjunction with herbicide-resistant crops to increase specificity of protection and efficiency. The availability of herbicides which can be applied on a broader range of crops may also result in the use of more environmentally-friendly herbicides which are less toxic to animals and to the environment (Gheysen *et al.*, 1989; Christou, 1994).

1.14 THE HERBICIDE BASTA

In recent years, the large agrochemical company AgrEvo (a subsidiary of Hoechst) has developed a non-selective contact herbicide effective against a wide range of monocotyledonous and dicotyledonous annual and perennial weeds. It is sold under the trade name Basta (also Buster, Finale and Ignite). The active ingredient of the herbicide is glufosinate-ammonium which is a racemate of D- and L-isomers of phosphinothricin (PPT) of which the L-form is the active one. Two alanine residues linked to a PPT moiety make up bialaphos (Bp) which is a natural tripeptide molecule produced by some strains of *Streptomyces* (Mazur and Falco, 1989; Product information obtained from AgrEvo).

Phosphinothricin is an analogue of glutamine that inhibits the amino acid biosynthetic enzyme, glutamine synthetase (GS) in bacteria and plants. This enzyme plays an important role in the assimilation of ammonia and the regulation of nitrogen metabolism in plants. Inhibition of this enzyme by PPT causes a rapid build-up of intracellular ammonia levels and an associated disruption of the chloroplast structure. This results in inhibition of photosynthesis and plant cell death. It is therefore clear that PPT has herbicidal properties (Mazur and Falco, 1989; Product information obtained from AgrEvo).

New herbicides can only be introduced onto the market after thorough testing. Factors that are considered are toxicity to mammals, fish and algae, effects on beneficial insects and soil organisms, the breakdown properties of the chemical and the metabolities that

are produced. The active ingredient of Basta is rapidly broken down so no contamination of the soil or groundwater occurs. Toxicological evaluations have shown that the use of the herbicide will not result in adverse effects to aquatic or terrestrial organisms. Even though PPT inhibits GS from bacteria, plants and mammals, its inability to cross the blood-brain barrier and its rapid clearance by the kidneys are the apparent reasons for its non-toxicity to mammals. An advantage of Basta is that it is a post-emergence herbicide and therefore weeds can grow and protect soil against erosion by wind and water until the crop is established. After application of Basta, the weeds die and form a mulch thereby further preventing soil erosion and also providing nutrients which can be released into the soil (Mazur and Falco, 1989; Product information obtained from AgrEvo).

Basta controls a wide range of grasses, sedges and broadleaf weeds which are a major problem in soya bean cultivation. It would be extremely useful to produce genetically engineered soya bean plants resistant to Basta. This can be accomplished by inserting a foreign gene that encodes the enzyme phosphinothricin N-acetyl transferase, into the plant nuclear DNA. The production of this enzyme by the transgenic plant would enable the plant to acetylate L-PPT in the presence of acetyl co-enzyme A, making the plant resistant to glufosinate-ammonium (Product information obtained from AgrEvo; Rosier, 1988).

1.15 AIMS OF THE STUDY

The aims of this investigation were to genetically engineer South African soya bean cultivars with a synthetic Basta resistance gene and to study aspects relating to the genetic engineering of plants from a theoretical as well as a practical point of view.

To develop an understanding of the broader issues relating to the genetic engineering of plants, a literature review was carried out. Topics covered included: the use of biotechnology to improve crop plants; the genetic engineering of plants with particular reference to recalcitrant crops such as soya bean; the importance of soya bean as a

crop; the use of herbicides in agriculture; and the production of herbicide resistant transgenic plants. In order to plan an experimental approach to genetically engineering South African soya bean cultivars, plant tissue culture and transformation techniques that have been applied to soya bean world-wide, were reviewed.

A practical study of plant tissue culture and transformation methods was conducted to develop an efficient, routine method for the regeneration of transgenic Basta resistant soya bean plants. As the study progressed, it became clear that the project was not as straightforward as initially anticipated. The aims were therefore broadened to include finding solutions to problems encountered such as *in vitro* shoot elongation, rooting and hardening off of *in vitro* regenerated plantlets. Because transgenic Basta resistant soya bean plants could not be produced, further studies on the non-recalcitrant crop, tobacco, which is a model system for plant genetic engineering were carried out. This allowed the optimization of assay systems for the analysis of transgenic plants containing the *pat* (Basta resistance) gene.

Molecular methods for the cloning of foreign genes into binary vectors suitable for plant genetic engineering were also investigated. Although constructs containing the *pat* gene were available, it was felt that a more suitable vector for research purposes should be constructed to contain the *pat* gene as well as the *uid*A (GUS reporter) gene for better detection of plant transformation.

CHAPTER 2

DEVELOPMENT OF TISSUE CULTURE TECHNIQUES FOR SOUTH AFRICAN SOYA BEAN CULTIVARS

2.1 INTRODUCTION

Soya bean (*Glycine max*) has been the object of considerable tissue culture research. Work on soya bean was first reported as early as 1961 when Miller studied soya bean cotyledon callus. Further research in this area was reported by Miller (1963), by Blaydes (1966), and by Feung *et al.* (1971). Also studied were soya bean root cells (Gamborg *et al.*, 1968; Gamborg and Finlayson, 1969). Gamborg *et al.* (1968) developed the well known B5 medium which is probably the second most widely used tissue culture medium after MS medium (Murashige and Skoog, 1962). Further work on root cells was reported by La Rue and Gamborg (1971), Miller *et al.* (1971) and Schenk and Hildebrandt (1972). Differentiation of plants from callus or from any other soya bean tissue in culture was not reported until 1973 when Kimball and Bingham found that adventitious buds formed on soya bean hypocotyl sections when cultured under certain conditions.

Further studies on soya bean included those of Gamborg *et al.* (1974) who worked on protoplasts, and Ivers *et al.* (1974) who worked on soya bean anther culture. Attempts were made by Beversdorf and Bingham (1977) and by Oswald *et al.* (1977) to regenerate soya bean plants. However, no plantlets were obtained from the callus cultures used. The first reports of successful soya bean plant regeneration were those using tissue cultures containing pre-existing meristems i.e. with cultures from cotyledonary nodes (Cheng *et al.*, 1980), stem nodes (Saka *et al.*, 1980) and shoot tips (Kartha *et al.*, 1981). Plants were also regenerated from cultures of the wild perennial

species *Glycine canescens* (Kameya and Widholm, 1981; Widholm and Rick, 1983; Grant, 1984) but the methods used were not applicable to cultivated soya bean.

Work on somatic embryogenesis in cell cultures of soya bean was reported by Phillips and Collins (1981), by Gamborg *et al.* (1983), by Christianson *et al.* (1983), and by Lippmann and Lippmann (1984). However, routine regeneration of fertile plants from embryogenic cultures was only reported later by Ranch *et al.* (1985), by Lazzeri *et al.* (1985), and by Ghazi *et al.* (1986). In 1986, plants were regenerated from callus derived from immature embryos via organogenesis or via embryogenesis depending upon the composition of the medium. Of note is the fact that fertile plants were obtained from all 54 genotypes tested (Barwale *et al.*, 1986). Fertile plants were produced via organogenesis by Wright *et al.* (1986) and Wright *et al.* (1987). Between 1987 and 1992, a large amount of work on somatic embryogenesis was carried out (Hammatt and Davey, 1987; Finer, 1988; Finer and Nagasawa, 1988; Lazzeri *et al.*, 1988; Buchheim *et al.*, 1989; Parrott, 1989; Wright *et al.*, 1991; Komatsuda *et al.*, 1992). Also during this time, soya bean plants were regenerated from protoplasts (Wei and Xu, 1988; Dhir *et al.*, 1991a; Dhir *et al.*, 1991b; Dhir *et al.*, 1992a).

There has been considerable research conducted on soya bean regeneration (further information is given in Chapter 4 where literature on regeneration of transgenic soya bean is discussed) but the crop has proved to be extremely recalcitrant to *in vitro* manipulation. This is thought to be due to the fact that the organizational level of plant cell and tissue cultures has a direct influence on the expression of morphogenetic competency. For example, cell cultures have proved recalcitrant to a wide range of regeneration protocols but plants can be readily generated from totipotent cells containing meristems (Lippmann and Lippmann, 1984; Ranch *et al.*, 1985; Dhir *et al.*, 1991a). Another problem with soya bean regeneration is that protocols are often genotype-dependent, particularly in the case of protoplasts (Dhir *et al.*, 1991a). Perhaps one of the most important problems with soya bean regeneration is that once shoots are obtained, they fail to produce roots readily. This makes regeneration unreliable and ineffective and makes cultivation of transgenic material impossible (Townsend and Thomas, 1994). This problem has been addressed and a method has

been patented for the production of roots on soya bean regenerants which makes use of the amino acid pyroglutamic acid and a hormone enriched medium (Townsend and Thomas, 1994).

The specific gene transfer systems to be used to produce transgenic plants must also be taken into account when choosing a regeneration protocol. With recalcitrant crops it is often advisable to develop a gene transfer system and a regeneration system in conjunction with one another. This is because not all cells of a plant can be transformed and not all cells can regenerate. The gene transfer system has to be directed to those cells which have the potential to regenerate. It must also be noted that leaf explants are usually poor starting material for transformation of recalcitrant crops like soya bean and that hypocotyls, cotyledons, embryos or shoot apices are more easily transformed. The choice of a regeneration protocol therefore depends on a large number of factors. These factors have to be taken into consideration, particularly if the aim is to obtain fertile transgenic plants.

In this chapter, the use of a variety of soya bean explants, tissue culture media and regeneration protocols are described. The successful regeneration of adventitious shoots from cotyledons of soya bean is discussed below, as well as methods for rooting of shoots and the routine production of fertile regenerated soya bean plants. A number of soya bean cultivars were tested in this study to ensure that the tissue culture methods developed were not genotype-dependent. There are always differences between cultivars with regard to *in vitro* response but it is necessary to develop tissue culture methods that result in *in vitro* regeneration of plants in most, if not all cultivars, for genetic engineering systems to be successful.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Seed of the *Glycine max* cultivars Forrest, Hutton, Kunitz, PNR494 and PNR577G was obtained from Pannar RSA (Pty) Limited, Greytown. Seed of the cultivars Ibis, Impala and Prima, and pods of Forrest, Hutton, Ibis, Impala, Prima and PNR494 were provided by the Oil and Protein Seed Centre, Department of Agricultural Development, Potchefstroom. Forrest and Hutton have a medium to short growing season and are widely adapted and therefore very useful. PNR494 and PNR577G are poor seed yielders while Ibis, Impala and Prima are high yielders. Kunitz has a short growing season but is high in trypsin which stunts animal growth. Ibis, Impala and Prima have long growing seasons and are best suited to warm conditions under irrigation. Prima belongs to Saffola while PNR494 and PNR577G are Pannar cultivars. Forrest, Hutton, Ibis. Impala and Kunitz are open cultivars and therefore require no special permission for experimentation (W. Goodwin, Personal Communication). Although all these cultivars were used in this study, the most suitable cultivars for the insertion of the herbicide resistance gene from an economic point of view were Forrest, Hutton, Ibis and Impala.

2.2.2 Plant tissue culture media

Plant tissue culture media used in this study are listed in Appendix A. All basal medium stock solutions were stored at 4°C without autoclaving. Vitamins (the organic supplement) were filter sterilized and stored at -20°C or 4°C. Plant growth regulators were made up as 1 mg/ml stocks, filter sterilized and stored at -20°C or 4°C. The agar, sucrose and basal medium were autoclaved at 121°C for 20 minutes. Unless otherwise stated, the vitamins and plant growth regulators were aseptically added to the medium following autoclaving. Media are written as the type of medium, the quantity of sucrose and where variable concentrations of hormones are used, the amounts per litre are

indicated in brackets eg. OR 20 (1) denotes OR medium containing 20 g/l sucrose and 1 mg/l of the variable plant growth regulator which in this case is BA. Unless otherwise stated, the pH of all tissue culure media was 5.7.

2.2.3 Surface sterilization of soya bean seeds and pods

In all experiments, the surface sterilization procedure was optimized by adding 0.001% Tween 20 to household bleach (JIK) containing 3.5% sodium hypochlorite diluted to the appropriate concentration. Following treatment, the plant material was rinsed six times in sterile distilled water to remove all traces of the sterilant.

2.2.3.1 Surface sterilization of soya bean seeds

Mature seeds were surface sterilized by soaking in either 1.75% or 3.5% sodium hypochlorite for 2 to 120 minutes or by soaking in 0.035% sodium hypochlorite for 24 hours. A pre-treatment method was also tested whereby seeds were soaked in 70% ethanol for one minute prior to soaking in sodium hypochlorite as detailed above.

2.2.3.2 Surface sterilization of soya bean pods

Immature seeds can be obtained from young soya bean pods which are still green. Immature seeds were sterilized using three surface sterilization approaches. Firstly, the sterilization of pods following the removal of surface hair with a scalpel blade or a razor blade and the subsequent dissection of the sterilized pods to obtain the immature seeds was tested. Secondly, the immature seeds were aseptically removed from surface sterilized pods and re-sterilized. Thirdly, seeds were surface sterilized following their non-sterile removal from pods. Either 3.5 or 1.75% sodium hypochlorite was used and exposure times varied between 2 minutes and 30 minutes.

2.2.4 Germination of soya bean seeds

Soya bean seeds were surface sterilized by soaking seeds in 3.5% sodium hypochlorite and 0.001% Tween 20 for two minutes. Seeds were either placed on tissue culture medium in Petri dishes (three seeds per plate, plates sealed with Parafilm), or on medium in 7.5 x 2.5 cm tubes, 125 ml tissue culture bottles or 250 ml tissue culture bottles (one seed per container). Seeds were germinated at 25°C or 28°C. The effect of light on germination was tested by placing some seeds in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) and other seeds in the dark. Seeds placed in the dark were transferred to the light after two to five days. Soaking seeds for 48 hours in sterile water containing 1 mg/l GA_3 to induce shooting (K. Lindeque, Personal Communication) prior to culture on germination medium was also tested.

Various tissue culture media (Appendix A) were tested for germination of soya bean seeds. These were MS 30, ½MS 30 (Murashige and Skoog, 1962), B5 30 and ½B5 30 (Gamborg *et al.*, 1968). The addition of 500 mg/l casein hydrolysate to ½MS 30 medium to improve growth (K. Lindeque, Personal Communication) and the addition of 0.5 or 1 mg/l BA to MS 30 or ½MS 30 medium (Appendix A) to improve shoot production upon regeneration (Wright *et al.*, 1986) was also tested.

Eight soya bean cultivars, namely Forrest, Hutton, Ibis, Impala, Kunitz, PNR494, PNR577G and Prima, were screened for differences in germination potential. Seeds of all eight cultivars were sterilized using optimized procedures and placed in Petri dishes on ½MS 30 medium to germinate. Seeds were placed in the dark at 25°C for two days and then transferred to the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$). After seven days, the number of seeds that were contaminated and the number of seeds that had germinated was recorded.

2.2.5 Regeneration from hypocotyls of soya bean

Soya bean seeds of the cultivars Forrest, Hutton, Ibis, Impala, Kunitz, PNR494, PNR577G and Prima were surface sterilized in 3.5% sodium hypochlorite containing 0.001% Tween 20. Seeds of all cultivars were sterilized for two minutes except in the case of the cultivar Kunitz where seeds were sterilized for five minutes due to higher levels of contamination. Seeds were rinsed in sterile distilled water and placed on ½MS 30 medium (Appendix A) to germinate. The sterilized seeds were either germinated in Petri dishes and used seven days later to provide explant material for regeneration experiments, or the seeds were germinated in 250 ml tissue culture bottles and used 14 days after sterilization.

After seven days on germination medium, hypocotyls of the cultivars Forrest, Hutton, PNR494 and PNR577G were cut into 1 cm lengths and placed in Petri dishes on ½MS or MS medium containing 0.5 or 1 mg/l BA, EB medium, EB B5 medium, MSC medium, B5 medium containing 0.5 mg/l BA and 1 or 1.5 mg/l 2,4-D, or MS containing 0, 1, 2 or 3 mg/l BA and 0.5, 2 or 4 mg/l NAA (Appendix A). Sucrose concentrations between 0 and 30 g/l were tested. Explants were placed in the dark or the light (16 hour phtoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) at 25°C or 28°C. Sixty hypocotyl segments of each cultivar were used to test each variable.

After seven days on germination medium, hypocotyl segments of the cultivars Forrest, Hutton, Ibis, Impala, Kunitz, PNR494, PNR577G and Prima were placed on MSC 20 medium (Appendix A) in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) at 25°C. Hypocotyl segments of these cultivars, seven or 14 days after germination were also placed in the light at 25°C on B5 BA 20 medium containing 0.5, 1, 2 or 3 mg/l BA, OR 20 medium containing 1, 2 or 3 mg/l BA, EB 20 medium containing 4, 8 or 12 mg/l NAA or ½MS 30 medium containing 1 mg/l BA (Appendix A). Sixty hypocotyl segments of each cultivar were used to test each variable.

Explants were assessed every seven days for their ability to produce shoots or to form callus *in vitro*. All explants were transferred to fresh medium every 28 days.

2.2.6 Regeneration from cotyledonary node meristems of soya bean

Soya bean seeds of the cultivars Forrest, Hutton, Ibis, Impala, Kunitz, PNR494, PNR577G and Prima were surface sterilized and germinated as described above in section 2.2.5. Additionally, seeds were primed by germination on ½MS medium containing 1 mg/l BA according to the method of Wright *et al.* (1987). Cotyledonary node meristems seven or 14 days after germination were obtained by removing cotyledons adjacent to the stem axis and cutting the stem and hypocotyl approximately 3 mm above and below the node region. Nodes were cultured in Petri dishes under the same conditions and on the same media as the hypocotyls (described above in section 2.2.5) and assessed in the same way for production of shoots or the formation of callus *in vitro*. Twenty cotyledonary node meristems of each cultivar were tested per variable.

2.2.7 Regeneration from leaf discs of soya bean

To obtain soya bean leaf material, surface sterilized seeds of the cultivar Forrest were germinated in 250 ml tissue culture bottles in the dark for five days and then transferred to the light for nine days i.e. a total of 14 days after germination. Leaves from the resulting seedlings were cut into 1 cm x 1 cm squares and placed in Petri dishes containing MSS 20, B5S 20, B5 BA 20 (2), OR 20 (1), MSR 20 or ½MS 30 medium supplemented with 1 mg/l BA (Appendix A) in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) at 25°C. For each variable, 20 leaf discs were used. Leaf discs were assessed every seven days for their ability to produce shoots *in vitro* and transferred to fresh medium every 28 days.

2.2.8 Regeneration from cell suspension cultures of soya bean

Cell suspension cultures of the cultivars Forrest, Hutton, PNR494 and PNR577G were established by excising hypocotyl segments from sterile seeds seven days after

germination. The hypocotyl segments were cultured on MSC 20 medium (Appendix A) in Petri dishes in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) at 25°C for four weeks to produce callus. The callus obtained was then placed in 20 ml liquid MSC 20 medium in 125 ml tissue culture bottles and shaker incubated at 100 rpm and 25°C to produce cell suspensions. Ten bottles were placed in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) and ten in the dark.

After 14 days, cell suspensions were placed in test tubes and allowed to separate into small, medium and large cell aggregates. Four ml of small cell aggregates were placed in 20 ml of liquid MSC 20 medium in 125 ml tissue culture bottles and left to shake at 100 rpm for 14 days to obtain fine cell suspensions. Cultures were maintained by removing 4 ml of cells and transferring into 20 ml of new liquid MSC 20 medium every 14 days.

Contamination was monitored by placing 1 ml of each cell suspension culture on solid MSC 20 medium in the light, tipping the Petri dishes to spread the cells and then removing excess liquid. The growth of contaminants, or the lack thereof, was assessed after seven days. Regenerability was tested by placing 1 ml of cells on OR 20 (1), MSR 20, B5 BA 20 (2) and ½MS 20 + 1 mg/l BA medium (Appendix A) in the light in the same way as above. Ten Petri dishes of each medium were used for each cultivar in this experiment. Cell suspension cultures were assessed for shoot production or callus formation every seven days.

2.2.9 Regeneration from embryonic axes of soya bean

Soya bean seeds of the cultivars Forrest, Hutton, PNR494 and PNR577G were sterilized for two minutes in 3.5% sodium hypochlorite containing 0.001% Tween 20. Embryonic axes were either excised from these seeds immediately i.e. no germination, or after the seeds had been germinated on ½MS 30 medium (Appendix A) for two, seven or 14 days. Embryonic axes were either placed in Petri dishes on EB 20 medium containing 4, 8 or 12 mg/l NAA or on OR 20 medium containing 1, 2 or 3 mg/l BA

(Appendix A) at 25°C in the light or in the dark. Twenty embryonic axes of each cultivar were used to test each variable. After four weeks, embryonic axes were transferred to MSR 20 or R5 20 medium (Appendix A) in the light at 25°C.

Also tested as explant material for regeneration were immature seeds obtained from immature pods. Pods were cut open and the green, immature seeds removed non-aseptically. The seeds were surface sterilized by soaking in 1.75% sodium hypochlorite containing 0.001% Tween 20 for five minutes and rinsed in sterile water as usual. Immature embryonic axes of the cultivars Forrest, Hutton, Ibis, Impala, PNR494 and Prima were removed from the seeds and placed on OR 20 medium containing 1. 3 or 5 mg/l BA, or EB 20 medium containing 4, 8 or 12 mg/l NAA (Appendix A) in the dark at 25°C. Twenty embryonic axes of each cultivar were used to test each variable. After four weeks, embryonic axes were transferred to either MSR 20 or R5 20 medium (Appendix A) in the light at 25°C.

Embryonic axes excised from seeds of all ages were assessed every seven days for the production of shoots or callus and transferred to fresh medium every 28 days.

2.2.10 Regeneration from cotyledons of soya bean

Seeds of the soya bean cultivars Forrest, Hutton, Ibis, Impala, Kunitz, PNR494, PNR577G and Prima were surface sterilized and germinated as described in section 2.2.5. Additionally, seeds were primed by germination on ½MS 30 medium containing 1 mg/l BA according to the method of Wright *et al.* (1987). After 0, 2, 7 and 14 days, cotyledons were either left whole or cut in half longitudinally and placed in Petri dishes adaxially or abaxially on the same media that the hypocotyls and cotyledonary node meristems were cultured on (section 2.2.5). Cotyledons were incubated in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) and in the dark at 25°C and at 28°C. One hundred and twenty cotyledons of each cultivar were used to test each variable.

Cotyledons from immature seeds were obtained from immature pods sterilized as described in section 2.2.9 above. Immature cotyledons of the cultivars Forrest, Hutton and PNR494 were cut in half longitudinally, placed on the same media as immature embryonic axes (section 2.2.9) and incubated in the same manner. Ten immature cotyledons of each cultivar were used to test each variable.

The mature and immature cotyledons were assessed every seven days for the production of shoots or callus and transferred to fresh medium every 28 days.

2.2.11 The production of whole rooted plants from soya bean shoots

In studies on the production of whole rooted soya bean plants, shoots regenerated from cotyledons isolated from seeds germinated for two days were used. Cotyledons were routinely incubated at 25°C in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) on B5 BA 20 (2) medium (Appendix A) for four weeks. However, the very short shoots (< 3 mm) produced using this method were unable to form roots *in vitro* due to very short or non-existent stems. Shoots of soya bean have to be approximately 1 cm in size to root and produce whole plants (Barwale *et al.*, 1986). Procedures for the elongation and rooting of soya bean shoots were therefore investigated. In all these studies, the cultivar Forrest was used. The sample sizes in the experiments are given in the tables in the results section. Once optimized systems were developed, the cultivars Hutton, PNR494 and PNR577G were tested to ensure that the systems used were genotype-independent.

2.2.11.1 Elongation of soya bean shoots

Unless otherwise stated, ½MS 30 medium (Appendix A) was used in all shoot elongation experiments. A number of factors affecting soya bean shoot elongation were investigated in this study. These included: the effect of container size and conformation (Petri dishes, 125 ml tissue culture bottles, 250 ml tissue culture bottles or Magenta

boxes) on the ability of shoots to elongate; the effect of an additional four weeks of culture on regeneration medium on shoot elongation; and the effect of the addition of supplements such as casein hydrolysate (500 mg/l), L-glutamine (500 mg/l) and asparagine (500 mg/l and 1000 mg/l) to the medium on the elongation of shoots. In all cases, soya bean shoot size and stem length were compared after four weeks incubation.

The effect of light intensities on regeneration and shoot elongation was studied by carrying out routine regeneration procedures for soya bean but cotyledons were incubated at 25°C in the dark, low light (16 hour photoperiod, 3.75 $\mu E.m^{-2}.s^{-1}$) or high light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$). After four weeks, shoots produced in each case were compared by measuring shoot size and stem length.

2.2.11.2 Rooting of elongated soya bean shoots

Various factors affecting rooting of soya bean shoots were studied. A variety of media, namely MS, B5, ½MS and ½B5 (Appendix A) were used to induce roots. Half MS medium was then used in further root induction experiments where gelrite (2 g/l), agar (8 g/l) and agarose (6 g/l) were tested as solidifying agents and media containing MS vitamins (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), or media lacking vitamins were compared. Sucrose concentrations of between 0 and 60 g/l and pH values between four and seven were tested. The plant hormones NAA, IBA, IAA, BA and GA_3 were used alone or in combination at concentrations of 0.1, 0.2 and 0.5 mg/l. Activated charcoal (1 g/l), vermiculite (20 g/l) and boron (4 g/l) were also tested for their ability to induce roots (G. Donn, K. Lindeque, J. Shyluk, Personal Communications). Sorbarods (Roberts and Smith, 1990) which are filter paper tubes (Figure 2.1) were also used in attempts to induce rooting. In all cases, shoot size, stem length and root production were compared after four weeks.

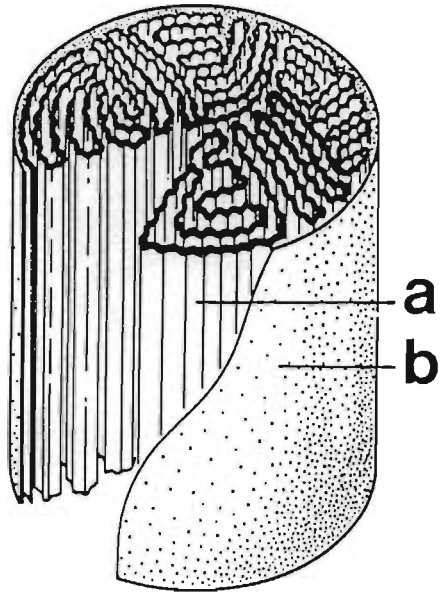

Figure 2.1 Schematic diagram of a sorbarod plug consisting of a filler of cold-crimped cellulose paper (a) wrapped with cellulose paper (b). (Roberts and Smith, 1990)

2.2.11.3 *In vitro* grafting of soya bean shoots

An *in vitro* method whereby the clumps of miniature shoots regenerated from cotyledons were grafted onto young seedlings of the same cultivar, in this case Forrest, was tested. To initiate grafting, seeds were surface sterilized and germinated *in vitro* for 14 days in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) at 25°C. The regenerated shoots were grafted onto these plantlets which were cut either above or below the cotyledons. A sorbarod (Roberts and Smith, 1990) was placed over the tip of the hypocotyl or epicotyl so that the cut edges just protruded. The clump of shoots was placed on top of the hypocotyl or epicotyl and held in place by the sorbarod (Figures 2.2 and 2.3). Twenty shoots were grafted onto the epicotyl and twenty shoots were grafted onto the hypocotyl. The grafted shoots were incubated at 25°C in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) for two weeks.

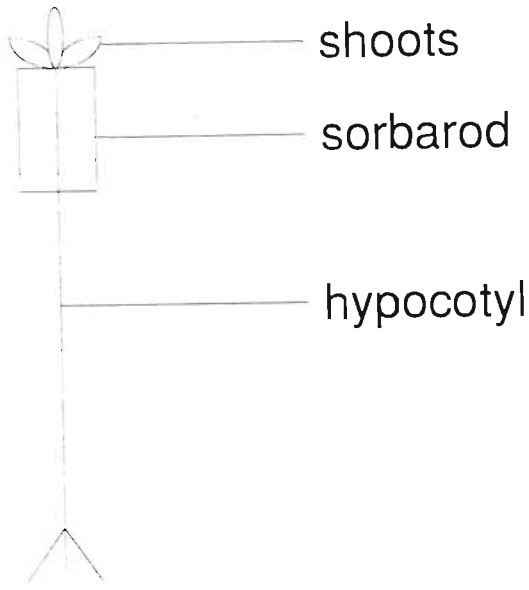

Figure 2.2 Grafting: schematic diagram of a soya bean seedling cut below the cotyledons

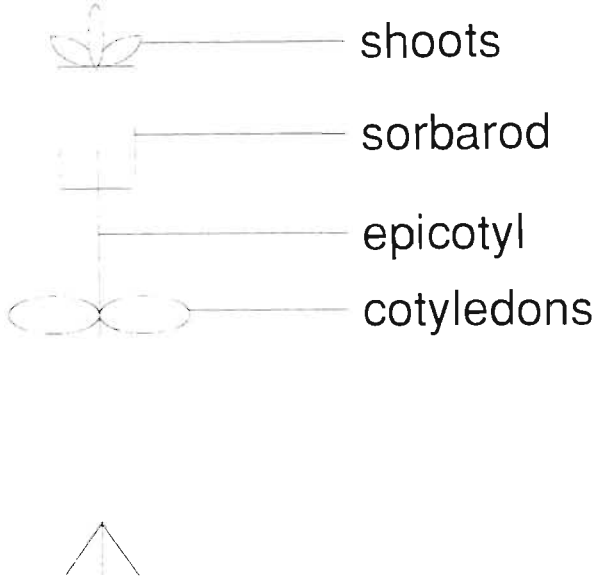

Figure 2.3 Grafting: schematic diagram of a soya bean seedling cut above the cotyledons

2.2.11.4 Hardening off of rooted soya bean shoots

The process of hardening off complete plantlets with well established roots and new shoot growth was initiated by loosening the lids of the tissue culture bottles for one week. After this, the plantlets were removed from the bottles and the agar was removed from the roots by rinsing in a 1 g/l Benlate (Dupont) solution. The plantlets were then placed in pots containing commercial potting soil. The potted plants were cultivated in either completely or partially sealed plastic bags (bottom end open). The bags were gradually opened (cutting off the corners and then the tops of the bags over three weeks) until the plants were accustomed to external conditions.

Because soya bean plantlets do not harden off well using the above method, another procedure was investigated whereby plantlets remained in *in vitro* culture until they produced pods.

A glasshouse was built recently at the CSIR and a mist bed constructed therein for hardening off plants. The mist bed was covered in plastic and shadecloth to create humid, shady conditions for the hardening off process. Temperature was controlled at 27°C but light intensity and day length in the glasshouse were dependent upon the time of year. Plants to be hardened off were placed in seed trays containing plastic seedling plugs filled with 1:1 mixtures of vermiculite and perlite, bark and perlite, potting soil and perlite, and potting soil and sand. The trays were placed in the mist bed and sprayed with water every ten minutes for 20 seconds. Over a period of two weeks, the cycle was lengthened until plants were being sprayed every 60 minutes for 20 seconds. The plants were also watered every three days with Hoagland's solution (Wetter and Constabel, 1982). After two to three weeks, the plants were transferred to pots containing commercial potting soil and approximately 10 g of 2:3:2 fertilizer per 15 cm pot. They were either left in the mist bed or moved onto benches in the glasshouse for further growth.

2.3 RESULTS

2.3.1 Surface sterilization of soya bean seeds and pods

2.3.1.1 Surface sterilization of soya bean seeds

It was found that the most effective method of surface sterilizing the seeds of all cultivars of soya bean tested was soaking in 3.5% sodium hypochlorite for two minutes. The exception was the cultivar Kunitz. This cultivar was very prone to fungal contamination and although sterilization has not been optimized for this cultivar, five minutes in disinfectant did provide enough clean material to work with. Lower concentrations of the sterilant, such as 0.035%, were not as effective at controlling contamination and longer contact times (five minutes to 24 hours) partially inhibited germination. Pre-treating seeds with ethanol reduced germination percentages. Ethanol appeared to damage seeds and resulted in pale, thin stems upon germination.

2.3.1.2 Surface sterilization of soya bean pods

Of the three methods used to surface sterilize soya bean pods, the most effective method (and also the easiest and quickest) was the non-sterile removal of immature seeds from pods and their subsequent soaking in 1.75% sodium hypochlorite for five minutes. It was found that the insides of pods were not necessarily sterile and even with hair removed it was difficult for the sterilant to come into contact with the surface of the pod because of air bubbles trapped thereon, so seeds removed from these pods required a further sterilization process. It was not necessary to surface sterilize the pods and then the seeds as contamination could be effectively controlled by the non-sterile removal of seeds from pods prior to the sterilization treatment.

2.3.2 Germination of soya bean seeds

Experiments conducted to optimize germination procedures showed that any containers could be used for the germination of seeds but all had advantages and disadvantages. Seeds germinated very well in Petri dishes but the rapid spread of contaminants resulted in many clean seeds becoming contaminated. Small (125 ml) tissue culture bottles were optimal as only one seed was placed in each bottle which resulted in no loss of clean seeds. However, when very young germinating seeds were required, Petri dishes were more practical as contamination had no time to spread. Large (250 ml) bottles were only useful when seedlings older than 14 days were required as the larger quantities of medium were otherwise wasted. Seeds germinated better at 25°C than at 28°C. At 28°C, the edges of the leaves of seedlings often turned yellow. If seeds were placed in the light to germinate, roots tended to grow towards the top of the container instead of into the medium. Orientating seeds differently in the medium did not solve the problem. Seeds placed in the dark for two to five days following sterilization produced roots which grew into the medium.

Screening of eight cultivars showed that there were definite differences in percentage germination and levels of contamination between cultivars as shown in Table 2.1. The results indicated that Forrest, Hutton, PNR494 and PNR577G were superior in both regards. Seed of Ibis, Impala and Prima was of poor quality (covered in soil, seed coats broken) which may account for the low germination percentages. The Kunitz seed used contained fungal contaminants which were difficult to eliminate and which could also have inhibited germination.

Table 2.1 Germination and contamination percentages obtained after screening eight soya bean cultivars for the effectiveness of surface sterilization procedures

Cultivar	% Germination of clean seed[a]	% Contamination
Forrest	93	2
Hutton	76	2
Ibis	45	12
Impala	62	8
Kunitz	40[b]	60
PNR494	88	5
PNR577G	72	1
Prima	53	10

a: Following surface sterilization of seeds using 3.5% sodium hypochlorite for two minutes

b: Following surface sterilization of seeds using 3.5% sodium hypochlorite for five minutes

The percentage *in vitro* germination of seeds was affected by the tissue culture medium used and by the inclusion of nutritional supplements and plant hormones in the medium. Use of MS 30 medium and in particular, ½MS 30 gave rise to healthy plants whereas B5 30 and ½B5 30 resulted in plants with yellow leaves. Supplementation of ½MS 30 medium with 500 mg/l casein hydrolysate had no beneficial effect on the plants. The addition of 0.5 or 1 mg/l BA to MS 30 or ½MS 30 medium resulted in abnormal seedlings with enlarged nodes, thickened stems and no roots. Priming of seeds by placing them on medium containing BA improved regeneration when the method of Wright *et al.* (1986) was used but not in other cases. Soaking seeds for 48 hours in GA_3 resulted in more leaves forming on seedlings but a lower percentage of seeds

germinated. This result confirmed previous results where soaking soya seeds in 0.035% sodium hypochlorite overnight inhibited germination.

Because of the findings discussed above, germinated seeds up to four days old were routinely cultured in Petri dishes, seedlings four to 14 days old were germinated and cultured in 125 ml bottles and seedlings older than 14 days in 250 ml bottles. All seeds were germinated on ½MS 30 medium at 25°C in the dark for two to five days before transfer to the light.

2.3.3 Regeneration from hypocotyls of soya bean

Soya bean shoots could not be regenerated from hypocotyls of any of the cultivars used under any of the conditions tested. However, hypocotyls placed on MSC medium produced large quantities of callus which grew very vigorously, doubling in size in two to three weeks. Callus growth was more vigorous at 25°C than at 28°C. Callus also grew faster in the light than in the dark. Hypocotyls produced very healthy, bright green callus when cultured in the light on as little as 2 g/l sucrose but could not be maintained for more than two months unless placed on higher concentrations of sucrose such as 20 g/l. There was no difference in response between explants used seven days after or 14 days after surface sterilization.

Hypocotyls placed on MSC 20 medium produced healthy callus within three weeks (Figures 2.4 and 2.5). However, cultivars responded differently. The cultivars Ibis, Impala and Prima produced large quantities of healthy callus. The cultivars Forrest, PNR494 and PNR577G produced slightly less. Callus from explants of the cultivars Hutton and Kunitz turned brown around the edges after five weeks.

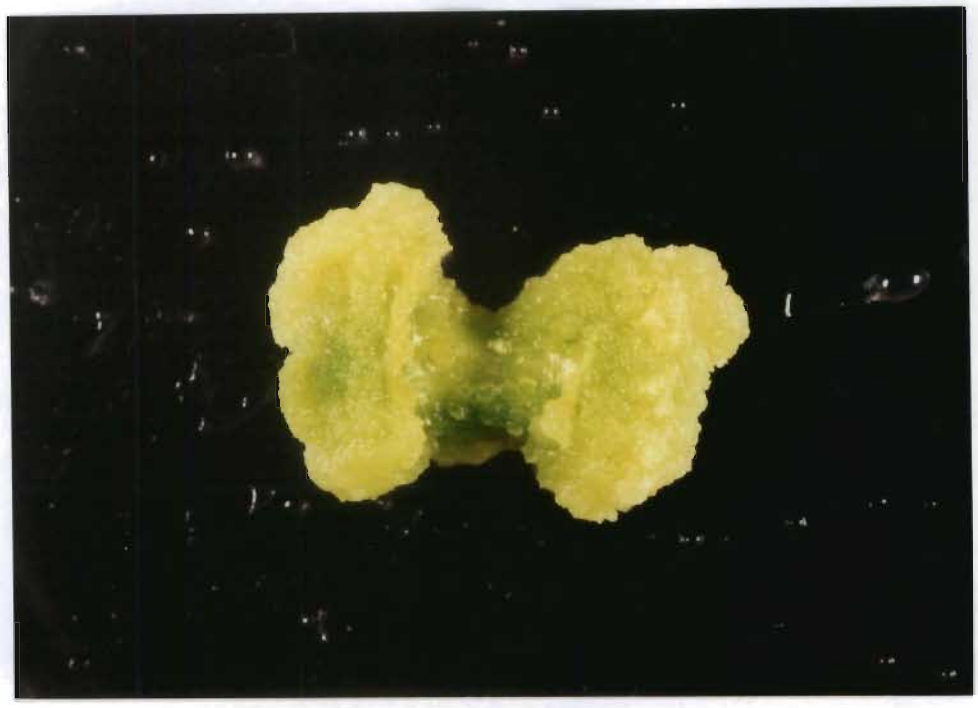

Figure 2.4 Callus produced from a hypocotyl segment of soya bean (cv. PNR577G) cultured on MSC 20 medium

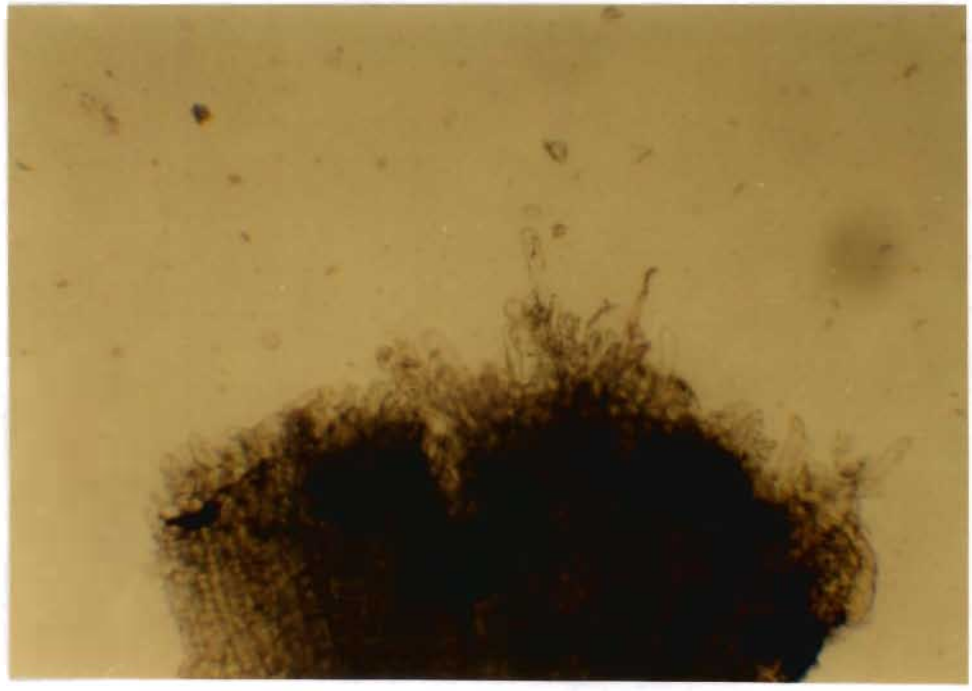

Figure 2.5 Elongating callus cells on the end of a soya bean (cv. PNR494) hypocotyl segment cultured on MSC 20 medium (280 X Mag.)

2.3.4 Regeneration from cotyledonary node meristems of soya bean

Shoots were produced from soya bean cotyledonary node meristems when nodes were placed on EB medium containing 12 mg/l NAA, B5 BA medium containing 1 mg/l BA and ½MS medium containing 1 mg/1 BA (Appendix A). Shoots were produced even when no sucrose was present in the medium and in fact lower concentrations of between 0 and 3 g/l sucrose appeared to enhance shoot production. In the case of EB and B5 BA media, only two to three shoots were produced per node. Nodes placed on ½MS medium containing 1 mg/l BA produced eight to ten shoots per node. It is important to note, however, that nodes placed on ½MS containing 1 mg/l BA only produced shoots if the nodes were excised from seeds primed on germination medium containing BA as described by Wright *et al.* (1986).

2.3.5 Regeneration from leaf discs of soya bean

Leaf tissue of the cultivar Forrest placed on B5S 20 and B5 BA 20 (2) medium turned yellow after seven days and brown after 14 days. Some black callus formed on the cut edges and wounds. Leaf discs on MSS 20, OR 20 (1) and MSR 20 medium remained green for five weeks and produced green callus on the cut edges and wounds but no shoots were produced. After 12 weeks, callus turned brown and the leaf discs were discarded.

2.3.6 Regeneration from cell suspension cultures of soya bean

Cell suspension cultures grown in the dark turned yellow and died while those in the light remained bright green. Occasionally, cultures became contaminated after extended incubation but when 1 ml of cells was plated out onto solid MSC 20 medium, contaminants were visible within one week. Cells plated out onto solid regeneration media grew into clumps of green callus (microcalli) within 14 days (Figures 2.6, 2.7 and 2.8) but microcalli did not develop further into plantlets. Although microcalli were

produced on all media tested, MSC 20 medium resulted in the largest, healthiest and fastest growing calli. No cultivar differences were observed.

Figure 2.6 **Soya bean (cv. Forrest) cell suspension culture plated out onto solid MSC 20 medium. The growth of calli can be seen**

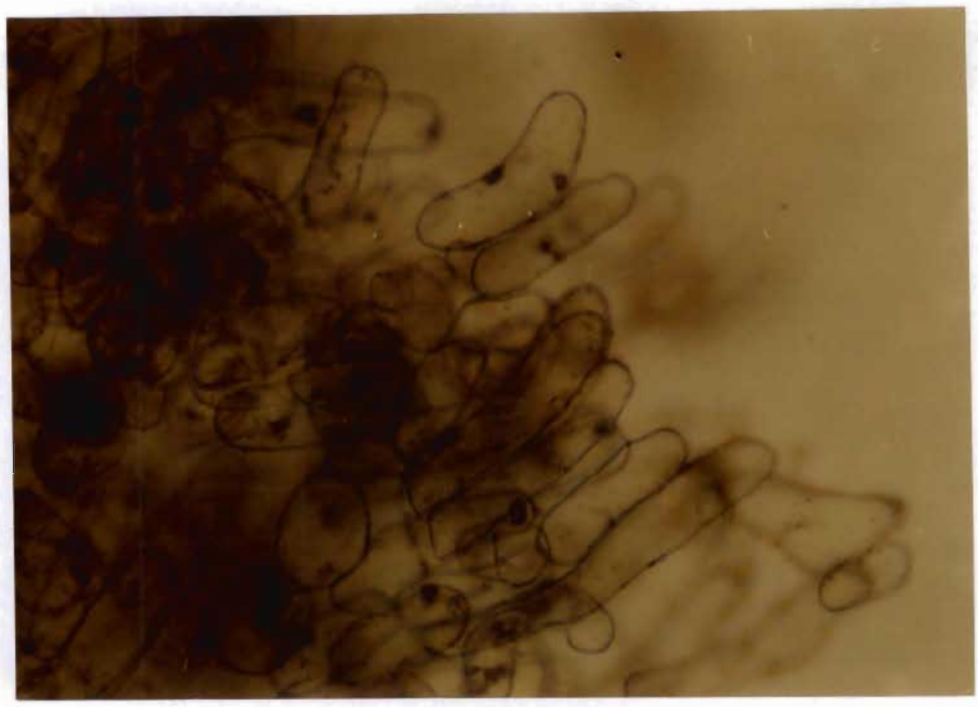

Figure 2.7 Soya bean (cv. Hutton) cell suspension culture showing elongated cells 14 days after initiation (870 X Mag.)

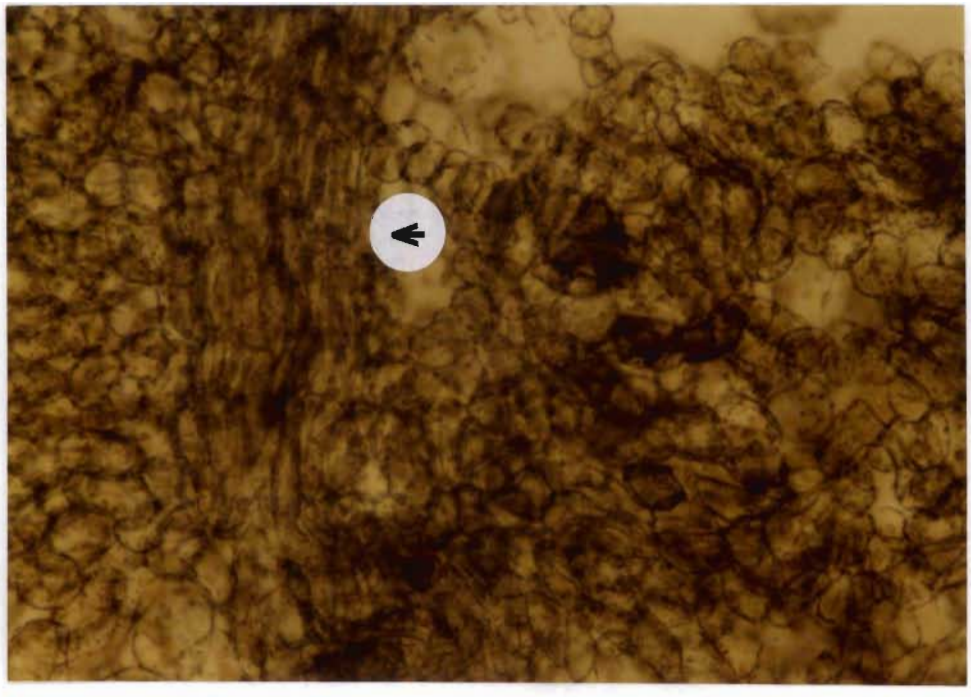

Figure 2.8 Cellular organization leading to vascular differentiation (arrow) of a soya bean (cv. PNR494) cell suspension culture plated out onto solid MSC 20 medium (870 X Mag.)

2.3.7 Regeneration from embryonic axes of soya bean

Embryos excised from seeds two, seven or 14 days after germination, from sterilized ungerminated seeds, and from immature seeds, produced shoots (Figure 2.9) in the fourth week of culture on MSR 20 medium, following initial culture on OR 20 medium for four weeks. Shoots were not produced when EB 20 or R5 20 media were used. Only three to five shoots per embryonic axis were produced in the case of embryonic axes isolated from ungerminated seeds. It was also difficult to cut open ungerminated seeds of the cultivars Hutton and PNR494 which made this method impractical. When embryonic axes were isolated from immature seeds and from seeds germinated for two days, eight to ten shoots per embryonic axis were produced. Embryonic axes isolated from immature seeds and from seeds germinated for two days were more responsive *in vitro* and more inclined to regenerate than material from ungerminated seeds and material from seven to 14 day old seedlings. Plant material from seeds germinated for two days was preferable to immature plant material however, because a store of dried seeds could be used. A regular supply of freshly grown field or glasshouse material was required in order to obtain immature material and this was found to be extremely difficult to arrange.

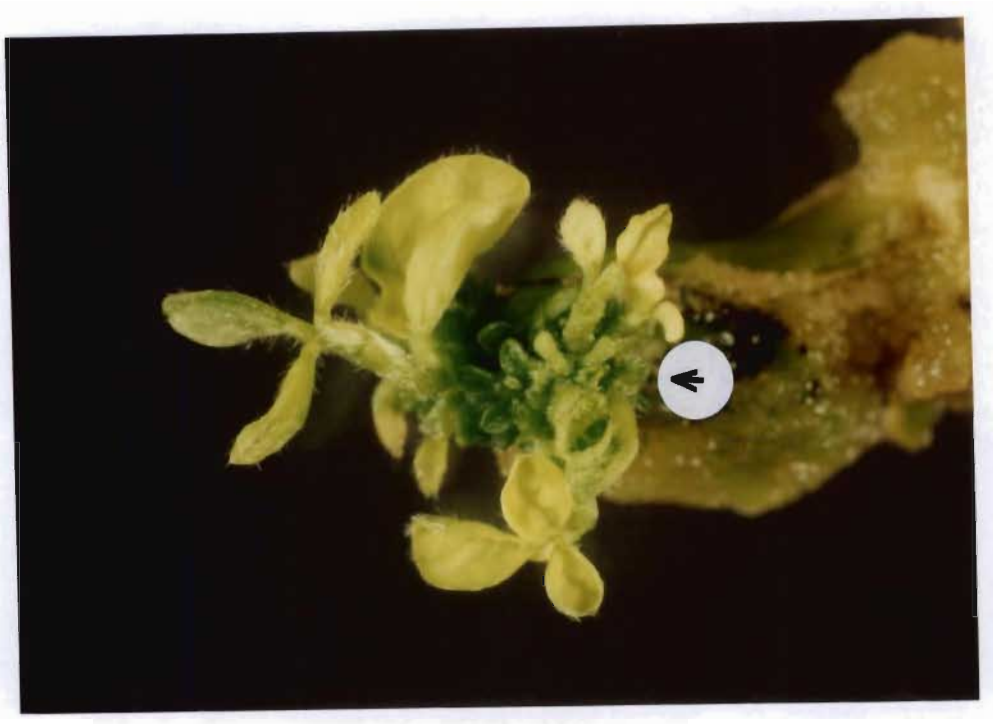

Figure 2.9 **Example of shoots (arrow) produced from a soya bean (cv. PNR494) embryonic axis. Regenerated soya bean shoots were produced from embryonic axes cultured on OR 20 medium in the dark for four weeks followed by culture on MSR 20 medium for a further four weeks under conditions of high light intensity. The embryonic axes were isolated from seeds that were germinated on ½MS 30 medium for two days**

2.3.8 Regeneration from cotyledons of soya bean

Efficient, routine adventitious shoot regeneration was obtained when cotyledons isolated from two day old seedlings were placed on B5 BA 20 medium containing 2 mg/1 BA in the dark or the light at 25°C (Figure 2.10). No shoots were produced on any of the other tissue culture media tested and priming of seeds by placing them on ½MS 30 medium containing 1 mg/l BA did not enhance regeneration. The age of explants was found to be vital for successful regeneration. Cotyledons excised from seeds germinated for two days regenerated but cotyledons from ungerminated seeds and from seven or 14 day old seedlings did not. Cotyledons produced shoots more readily when placed abaxial side down on the medium than when placed adaxial side down.

Immature cotyledons excised from immature pods, unlike cotyledons excised from seeds germinated for two days, produced some shoots when cultured on OR 20 medium followed by MSR 20 medium but very rarely (0.1%). This indicated that cotyledons isolated from immature seeds were very responsive in tissue culture but cotyledons isolated from seeds germinated for two days were preferred due to the ease of obtaining material to work with.

Figure 2.10 Example of shoots (arrow) produced from a soya bean (cv. Forrest) cotyledon cultured in the light for four weeks. Regenerated soya bean shoots were produced from cotyledons cultured on B5 BA 20 (2) medium. The cotyledons were isolated from seeds that were germinated on ½MS 30 medium for two days

2.3.9 The production of whole rooted plants from soya bean shoots

2.3.9.1 Elongation of soya bean shoots

The effects of various factors on shoot elongation of the cultivar Forrest are shown in Tables 2.2, 2.3 and 2.4. The use of Magenta boxes increased the size of shoots slightly but had no effect on the length of stems. All other containers had no effect on shoot elongation (Table 2.2).

Table 2.2 Effect of container type on soya bean (cv. Forrest) shoot size and stem length *in vitro*[a]

Container	Sample size (no. shoots)	Size of shoots (mm)	Length of stems (mm)
Petri dishes	4000	1 - 3	0.1
125 ml tissue culture bottles	500	1 - 3	0.1
250 ml tissue culture bottles	500	1 - 3	0.1
Magenta boxes	400	3 - 5	0.1

a: B5 BA 20 (2) regeneration medium used

Culturing cotyledons on B5 BA 20 (2) medium for eight weeks instead of four, increased the percentage of shoots obtained by 5% but shoot quality decreased dramatically. The additional culture period had no effect on shoot size and stem length (Table 2.3).

Table 2.3 Effect of additional culture period on soya bean (cv. Forrest) shoot size and stem length *in vitro*

Time	Sample size (no. shoots)	Size of shoots (mm)	Length of stems (mm)
Explants on B5 BA 20 (2) medium for four weeks	4000	1 - 3	0.1
Explants on B5 BA 20 (2) medium for eight weeks	400	1 - 3	0.1

The addition of supplements to the regeneration medium had no effect on shoot elongation and only a slight effect (2 to 4 mm versus 1 to 3 mm) on shoot size when both casein hydrolysate and L-glutamine were added to the medium (Table 2.4).

Table 2.4 Effect of tissue culture medium supplementation on soya bean (cv. Forrest) shoot size and stem length *in vitro*[a]

Medium	Sample size (no. shoots)	Size of shoots (mm)	Length of stems (mm)
Without additions	4000	1 - 3	0.1
With 500 mg/l casein hydrolysate	500	1 - 3	0.1
With 500 mg/l L-glutamine	500	1 - 3	0.1
With 500 mg/l asparagine	500	1 - 3	0.1
With 1000 mg/l asparagine	500	1 - 3	0.1
With 500 mg/l casein hydrolysate and 500 mg/l L-glutamine	500	2 - 4	0.1

a: B5 BA 20 (2) regeneration medium used

The effect of light intensities on soya bean shoot regeneration and shoot elongtion is shown in Tables 2.5 and 2.6. Almost 20% more shoots were produced in the dark than in the light. These shoots were also much larger (5 to 10 mm in size with stems 10 to 50 mm in length) than those produced in the light. Differences in elongation can be observed by comparing Figures 2.10 (in section 2.3.8), 2.11 and 2.12.

Table 2.5 Effect of light intensities on the frequency of soya bean (cv. Forrest) shoot regeneration *in vitro*[a]

Light conditions	Sample size (no. cotyledons)	% Regeneration[b]
High (37.5 $\mu E.m^{-2}.s^{-1}$)	2000	45
Low (3.75 $\mu E.m^{-2}.s^{-1}$)	400	45
Dark	1000	62

a: B5 BA 20 (2) regeneration medium used

b: percentage of cotyledons which produced shoots *in vitro*

Table 2.6 Effect of light intensities on soya bean (cv. Forrest) shoot size and stem length *in vitro*[a]

Light conditions	Sample size (no. shoots)	Size of shoots (mm)	Length of stems (mm)
High (37.5 $\mu E.m^{-2}.s^{-1}$)	2000	1 - 3	0.1
Low (3.75 $\mu E.m^{-2}.s^{-1}$)	400	1 - 4	1
Dark	1000	5 - 10	10 - 50

a: B5 BA 20 (2) regeneration medium used

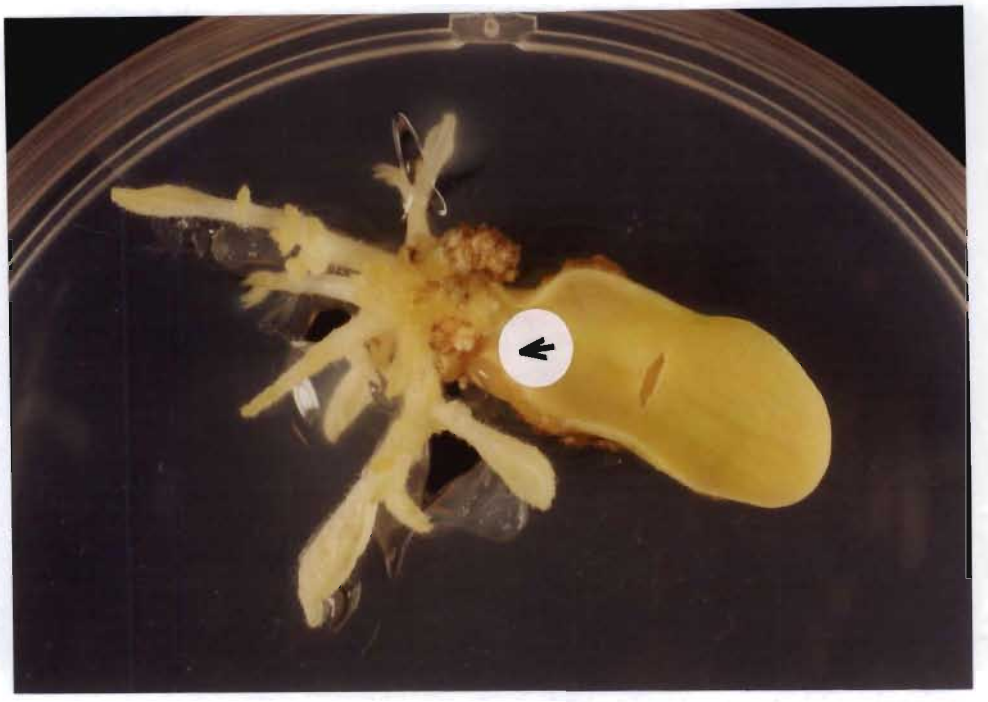

Figure 2.11 Example of shoots (arrow) produced from a soya bean (cv. Forrest) cotyledon cultured in the dark for four weeks. These regenerated soya bean shoots, etiolated due to culture in the dark, were produced from cotyledons cultured on B5 BA 20 (2) medium. The cotyledons were isolated from seeds that were germinated on ½MS 30 medium for two days

Figure 2.12 Example of shoots (arrow) produced from a soya bean (cv. Forrest) cotyledon cultured in the dark for four weeks and then transferred to conditions of high light intensity for seven days. Regenerated soya bean shoots were produced from cotyledons cultured on B5 BA 20 (2) medium. The cotyledons were isolated from seeds that were germinated on ½MS 30 medium for two days

2.3.9.2 Rooting of elongated soya bean shoots

The effects of various factors on root induction are shown in Tables 2.7, 2.8, 2.9, 2.10, 2.11, 2.12 and 2.13. The best medium for root production was ½MS medium with MS vitamins, 30 g/l sucrose, pH 5.7 solidified with agar. Sorbarods soaked with ½MS liquid medium containing MS vitamins and 30 g/l sucrose also yielded good results. The use of B5 30 or ½B5 30 medium resulted in shoots turning brown and very few roots being produced (Table 2.7).

Table 2.7 **Effect of tissue culture medium composition on the frequency of *in vitro* root production when soya bean (cv. Forrest) shoots were produced in the light[a]**

Medium	Sample size (no. shoots)	% shoots producing roots
MS 30	300	17
B5 30	300	3
½MS 30	4000	23
½B5 30	300	5

a: shoots 1-3 mm in size, stems 0.1 mm long used

The use of ½MS 30 medium with no vitamins or with B5 vitamins added resulted in browning of shoots and fewer roots being produced than in the case of ½MS 30 medium containing MS vitamins (Table 2.8).

Table 2.8 **Effect of vitamins in the tissue culture medium on the frequency of *in vitro* root production in soya bean (cv. Forrest)[a]**

Vitamins[b]	Sample size (no. shoots)	% shoots producing roots
None	300	28
B5	300	44
MS	4000	57

a: shoots 1-3 mm in size, stems 0.1 mm long used

b: vitamins added to ½MS 30 medium

Between 10 and 30 g/l sucrose was optimal for root induction with 30 g/l producing the best results. Concentrations of 0 and 60 g/l sucrose resulted in very low percentages of shoots producing roots (8 and 4% respectively) (Table 2.9).

Table 2.9 Effect of sucrose concentration in the tissue culture medium on *in vitro* root production in soya bean (cv. Forrest)[a]

Sucrose concentration[b] (g/l)	Sample size (no. shoots)	% shoots producing roots
0	100	8
1	100	25
3	100	45
5	100	43
10	200	50
15	200	51
20	200	53
30	500	61
40	200	25
60	100	4

a: shoots 1-3 mm in size, stems 0.1 mm long used

b: sucrose added to ½MS medium

Low pH values (4 to 5.5) of the tissue culture medium resulted in the cut edges of the stems turning black and very few roots were produced (7 to 21%). At pH values above 6, very few roots were produced and 50% of those shoots that did root, flowered spontaneously *in vitro*. At a pH of 5.7, only 23% of shoots produced roots but these plantlets were healthy (Table 2.10).

Table 2.10 Effect of pH of the tissue culture medium on the frequency of *in vitro* root production in soya bean (cv. Forrest)[a]

pH[b]	Sample size (no. shoots)	% shoots producing roots
4	100	21
5	100	8
5.5	100	7
5.7	4000	23
6	100	8
7	100	0

a: shoots 1-3 mm in size, stems 0.1 mm long used

b: pH of ½MS 30 medium

In most cases, when plant hormones were added to the tissue culture medium, roots were produced from the callus that formed on the cut edges of the stems where they had been removed from the cotyledons (Figure 2.13). In all cases, plantlet development failed to occur. The effect of plant hormones on soya bean root production is shown in Table 2.11. There were no differences in plant response to the different concentrations of plant hormones used.

Figure 2.13 Roots produced from callus growing on the cut edge of a soya bean (cv. Forrest) shoot removed from a cotyledon and cultured on ½MS 30 medium containing 0.1 mg/l IAA. Shoots with these type of roots did not develop further

Table 2.11 Effect of plant hormones in the tissue culture medium on the frequency of *in vitro* root production in soya bean (cv. Forrest)[a]

Plant hormone[b]	Sample size (no. shoots)	% shoots producing roots[c]
NAA	100	17
IBA	50	4
IAA	100	75
NAA + IBA	50	71
NAA + IAA	50	75
IBA + IAA	50	58
NAA + IBA + BA	50	0
NAA + IAA + BA	50	0
IBA + IAA + BA	50	7
GA_3	50	21

a: shoots 1-3 mm in size, stems 0.1 mm long used

b: plant hormones added to ½MS 30 medium at concentrations of 0.1, 0.2 and 0.5 mg/l

c: average of all three plant hormone concentrations - between the three hormone concentrations used, there were no significant differences in the percentage of shoots producing roots

The addition of activated charcoal to the medium resulted in vitrification of shoots. Vermiculite inhibited root production and addition of boron resulted in shoots being bleached. The use of sorbarods increased the percentage of shoots that produced roots (Table 2.12).

Table 2.12 Effect of treatment on the frequency of *in vitro* root production in soya bean (cv. Forrest)[a]

Treatment[b]	Sample size (no. shoots)	% shoots producing roots
Activated charcoal (1 g/l)	50	35
Vermiculite (20 g/l)	50	6
Boron (4 g/l)	50	0
Sorbarods[c]	1000	68

a: shoots 1-3 mm in size, stems 0.1 mm long used

b: added to solid ½MS 30 medium

c: added to liquid ½MS 30 medium

A dramatic increase in the frequency of root production was observed when shoots regenerated from cotyledons incubated in the dark were used for rooting. Also, although a higher percentage of shoots rooted in solid medium (88%) than in sorbarods (72%) (Table 2.13), healthier whole plants were produced in sorbarods. More shoots developed and roots did not turn brown as they did when cultured on solid medium (Figures 2.14 and 2.15). In both cases, some roots were produced within two weeks but usually four weeks were required for shoots to develop into whole rooted plants which could be hardened off.

Table 2.13 Effect of support medium on the frequency of *in vitro* root production when soya bean (cv. Forrest) shoots were produced in the dark[a]

Support medium	Sample size (no. shoots)	% shoots producing roots
Solid ½MS 30	200	88
Sorbarods[b]	200	72

a: shoots 5-10 mm in size, stems 10-50 mm long used

b: soaked with ½MS 30 liquid medium

Figure 2.14 Soya bean (cv. Forrest) plantlet rooting *in vitro* in a sorbarod soaked with ½MS 30 liquid tissue culture medium

Figure 2.15 Soya bean (cv. Forrest) plantlet rooting *in vitro* in solid ½MS 30 tissue culture medium. Note the browning of roots

Although all elongation and rooting experiments were conducted using the cultivar Forrest, optimized methods involving regeneration in the dark and rooting of regenerated shoots on solid ½MS 30 medium or in sorbarods were also tested on the cultivars Hutton, PNR494, and PNR577G to ensure that the methods developed were genotype-independent. Whole rooted plants were successfully produced in all cases.

2.3.9.3 *In vitro* grafting of soya bean shoots

The *in vitro* grafting method proved to be of no use in the elongation of soya bean shoots of the cultivar Forrest. When seedlings were cut below the cotyledons, the seedlings died and the clump of regenerated shoots attached to the hypocotyl did not develop further. When seedlings were cut above the cotyledons, the seedlings continued to grow and pushed the clump of shoots off the epicotyls so that no further development of the shoots occurred.

2.3.9.4 Hardening off of rooted soya bean shoots

When plantlets were placed in completely sealed plastic bags for hardening off, moisture gathered on the inside of the bags and a fungus developed on the plants. When partially sealed bags were used, no moisture accumulated and plants hardened off to some extent even before the corners and the tops of the bags were opened. Figure 2.16 shows a plant that has been hardened off using the latter method and from which seeds were collected. These seeds are currently undergoing field testing to ensure that the tissue culture manipulations have not adversely affected seed production and that progeny are normal.

Figure 2.16 Hardened off soya bean (cv. PNR577G) plant. This plant was produced by hardening off in a partially sealed plastic bag

Soya bean plantlets cultured in tissue culture bottles for two months flowered and produced pods *in vitro* from which seeds were collected (Figure 2.17). This is a useful characteristic as the plants do not have to be hardened off in order to collect seed material. A disadvantage is that usually only one or two pods containing a single seed each are produced per plant. In the case of hardened off soya bean plants, five to 15 pods containing two to three seeds are produced per plant depending upon the cultivar used.

Figure 2.17 Soya bean (cv. Forrest) plant flowering and producing pods *in vitro*

The use of a mist bed greatly improved the number of soya bean plants which survived the hardening off process (Table 2.14). The plants hardened off in the mist bed also produced more pods (10 to 15 pods) than the plants hardened off in plastic bags (5 to 10 pods) or those that produced pods *in vitro* (1 to 2 pods), especially if left in the mist bed instead of being placed on benches in the glasshouse. The recovery of plants from the hardening off process was the same in all soil mixtures used and, once established, plants grew equally well in the different potting mixtures (Figure 2.18).

Table 2.14 Effect of treatment on the recovery of hardened off soya bean plants (cv. Forrest)

Treatment	Sample size (no. plants)	% Recovery[a]
Sealed bags	60	2
Partially sealed bags	60	5
In vitro	40	1[b]
Mist bed	30	95

a: indicates plants that survived hardening off, flowered and produced pods

b: indicates plants that produced pods *in vitro* without hardening off

Figure 2.18 Hardened off soya bean (cvs. Forrest and Hutton) plants in a plastic and shadecloth-covered mist bed. The use of the mist bed greatly improved the hardening off process

2.4 DISCUSSION

Surface sterilization of explant material is of vital importance in tissue culture as contaminated material usually fails to grow or regenerate *in vitro*. In most cases, soya bean seed contaminants were bacterial. Fungal contamination of seeds was usually due to non-sterile technique and could be eliminated by more careful techniques. Seed of the cultivar Kunitz, however, had a persistent fungal contamination problem which could not be eliminated by more stringent surface sterilization treatments. Antibiotics can be included in the tissue culture medium to reduce contamination but as 80 to 100% of the

soya bean seeds were free of contamination using procedures developed in this study, this precaution was unnecessary and not worth the expense or the risk of antibiotic damage to the plant material. As long as sterile seeds could be distinguished from non-sterile seeds *in vitro* before lengthy experimentation was carried out, a small percentage of contaminated seeds was considered acceptable.

It was found that soaking seeds overnight in 0.035% sodium hypochlorite had an inhibitory effect on germination in soya bean and seed contamination could not be controlled. Ethanol aids penetration of sodium hypochlorite into seeds (George and Sherrington, 1984) but the seeds are more easily damaged by subsequent sodium hypochlorite treatment. Germination of soya bean seeds was adversely affected by ethanol treatment. The most effective method of sterilizing soya bean seeds (in seven out of eight cultivars) was soaking seeds in 3.5% sodium hypochlorite for two minutes followed by extensive rinsing in sterile water.

From the surface sterilization experiments conducted using pods, it was shown that the insides of pods are not sterile and that the immature seeds inside require a sterilization procedure. Sterilization of immature seeds can be satisfactorily accomplished following the non-sterile removal of the seeds from the pods. From the results obtained in this study, it is clear that the less time spent in the sterilant, the less damage caused to the mature or immature seed. This is important because damage to the seeds results in a decrease in the germination potential of the seeds.

Several factors were found to influence the *in vitro* germination of soya bean seeds. The choice of container for germination depended largely on the age of the seedling required for experimentation; the older the seedling desired, the larger the container required. The average temperature used in tissue culture experiments is 25°C (George and Sherrington, 1984) and it was shown that this temperature was also suitable for the germination of soya bean seeds. As seeds germinated in the field are placed in the dark for the first few days (in soil), it was found that, as expected, seeds germinated in tissue culture performed best under the same conditions i.e. dark for two to five days.

Of the germination media tested, ½MS 30 medium gave rise to the healthiest plants and was thus used routinely.

Although the use of germination medium lacking hormones results in healthy plants, Wright *et al.* (1986) found that if seeds were not germinated on medium containing BA. cotyledonary node meristems could not produce multiple shoots or form buds and this has been confirmed in this study. When cotyledonary nodes were used as explants for shoot growth from meristems, seeds were therefore routinely germinated on ½MS 30 medium containing 1 mg/l BA. The inclusion of 1 mg/l BA in the tissue culture medium did not enhance regeneration when cotyledons were used as explants for adventitious shoot production from the epidermal layers of cotyledons. The same number of shoots were produced per cotyledon without seeds being primed on medium containing BA.

Regeneration *in vitro* involves optimizing many factors such as the type of explant, the age and size of the explant, the temperature and the light conditions for culture, the tissue culture medium used, the concentration of inorganic components in the medium, the sucrose concentration, and the hormones used, and their concentrations. All these factors were tested in this study and optimized for soya bean. The conditions and media components listed above differed according to the type of explant used. Only the optimal temperature (25°C) at which cultures were incubated was constant in all cases.

After investigating soya bean hypocotyls as a possible explant for regeneration, it was found that adventitious shoots could not be regenerated from hypocotyls on any of the tissue culture media tested in this study. However, large quantities of actively growing callus could be obtained when hypocotyls were placed on MSC 20 medium in the light. The ability to produce callus from explants of soya bean is useful in a transformation system because of the information that can be obtained (Hinchee *et al.*, 1988; Luo *et al.*, 1994). Various *Agrobacterium* strains can be used to transform hypocotyls to determine which strains are able to infect soya bean tissues. The transgenic soya bean hypocotyl callus can then also be used to optimize various assay systems, such as PCR and DIG-labelling, for the analysis of transgenic soya bean.

Although shoots were produced from cotyledonary node meristems of soya bean cultured in the light, the shoots were derived from existing meristems and were not regenerated from *de novo* produced meristems. This means that they had already differentiated prior to placement on tissue culture medium. This makes the nodes unsuitable explants for genetic engineering because, ideally, a single cell should be transformed and allowed to regenerate into a plantlet to produce a completely transgenic plant. In the case of the transformation of cotyledonary node meristems, it is expected that chimaeric plants would be produced due to the multicellular nature of the existing meristems.

Leaf discs and cell suspension cultures of soya bean did not regenerate into plantlets on any of the tissue culture media tested. However, cell suspension cultures plated out onto MSC 20 medium in the light produced large, actively growing calli. Cell cultures can be useful in transformation studies because it is relatively easy to obtain transient gene expression in these cultures (personal experience with potato cell cultures).

Regenerated soya bean shoots were produced from embryonic axes cultured on OR 20 medium in the dark for four weeks followed by culture on MSR 20 medium in the light for four weeks. The frequency of shoot production, however, was fairly low (± 30%) and this method was therefore not used routinely to produce regenerated soya bean shoots.

Routine, highly efficient (± 70%) regeneration of soya bean shoots was obtained when cotyledons excised from seeds germinated for two days were used as explants and cultured on B5 BA 20 medium containing 2 mg/l BA. It was found that the age of the cotyledons was of critical importance in allowing regeneration from these explants and that plant material seven or 14 days after germination lost the potential to regenerate.

The important problems of *in vitro* soya bean shoot elongation, rooting of soya bean shoots and hardening off of soya bean plants have been overcome in this study. Initially, regenerated shoots were obtained but very few hardened off plants could be produced because of these problems. Regeneration is now routinely carried out in the dark. This type of culture results in shoots 5 to 10 mm in size and unusually long stems,

10 to 50 mm in length. The shoots are then able to form roots *in vitro* in sorbarods or in solid ½MS 30 medium. All other factors tested in this study proved to be of no importance in promoting shoot elongation or in inducing root production. The use of a mist bed has eliminated the problems associated with the hardening off of soya bean plants. It is now routinely possible to obtain hardened off fertile regenerants from soya bean cotyledons isolated from seeds germinated for two days in a genotype-independent fashion.

CHAPTER 3

GENE MANIPULATION FOR THE TRANSFER OF BASTA RESISTANCE TO SOUTH AFRICAN SOYA BEAN CULTIVARS

3.1 INTRODUCTION

The herbicide Basta contains the active ingredient glufosinate-ammonium which is made up of D- and L- isomers of phosphinothricin (PPT). L-phosphinothricin inhibits the enzyme glutamine synthetase which plays an important role in the assimilation of ammonia and the regulation of nitrogen metabolism in plants. It is possible to genetically engineer plants to be resistant to the herbicide Basta. This can be accomplished by inserting a foreign gene that encodes the enzyme phosphinothricin N-acetyltransferase, into the plant nuclear DNA. The production of this enzyme by the transgenic plant enables the plant to acetylate L-PPT in the presence of acetyl co-enzyme A, making the plant resistant to glufosinate-ammonium (Rosier, 1988; Product information obtained from AgrEvo).

Phosphinothricin is a natural molecule produced by at least two *Streptomyces* species which contain highly efficient phosphinothricin resistance genes. One research group, working on contract to Meiji Seika at Biogen, discovered the bialaphos resistance gene, *bar*, in the genome of *Streptomyces hygroscopicus*. Another group at Bielefeld University and in co-operation with AgrEvo AG discovered the phosphinothricin-acetyltransferase gene, *pat*, from *Streptomyces viridochromogenes* (Donn, 1991).

Both the *pat* and *bar* genes convert L-phosphinothricin into N-acetylphosphinothricin which does not inhibit glutamine synthetase. The *pat* and *bar* genes both contain 70% guanine-cytosine pairs in the DNA sequence while typical plant genes have only 50% G-C pairs. Because of the possibility that plant cells could inactivate the *Streptomyces* gene by methylating the cytosine bases, AgrEvo designed a synthetic version of the *pat*

gene with a lower G-C content. The gene codes for the same protein but consists of codons preferred by plants (Donn, 1991).

The *pat* gene was obtained from AgrEvo on five plasmids, namely pOCA/Ac, pB2/35SAcK, pHoe6/Ac, pHoe106/Ac and pHoe200/Ac (Appendix C). Although the *pat* gene itself is a selectable marker it is often useful to have a screenable reporter gene such as the β-glucuronidase (GUS) reporter gene (*uid*A) to follow transformation events. This provides an early detection system for successful transformation and allows the monitoring of the presence of transformed tissue. Ideally, for commercial purposes, only one or two foreign genes of interest should be present in the plant DNA. It is a disadvantage to have the GUS reporter gene present in plants produced on a commercial scale but, for research purposes, it is extremely beneficial. For this reason it was decided to subclone the *pat* gene into the binary plant vector pBI121 which contains the GUS reporter gene *uid*A (Appendix C).

The vector pBI121 was chosen as the plasmid in which to insert the *pat* gene because it has been used successfully to transform a wide variety of plants (Jefferson *et al.*, 1987; Bekkaoui *et al.*, 1988; Hodel *et al.*, 1992). Also of note is that other plasmids containing the combination of the *npt*II gene and the GUS reporter gene, both of which are present in pBI121, have been used successfully in the transformation of soya bean (Hinchee *et al.*, 1988; McCabe *et al.*, 1988). The sub-cloning of the *pat* gene into the plant vector pBI121 is described below.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

Bacteria and plasmids used in DNA manipulations and transformation experiments are shown in Tables 3.1, 3.2 and 3.3. Maps of the plasmids pOCA/Ac, pB2/35SAcK, pHoe6/Ac, pHoe106/Ac, pHoe200/Ac, pBI121, pJIT119 and pCNL35 are given in

Appendix C. All strains were maintained on Luria agar (LA) (Appendix B) containing the relevant antibiotics. *E. coli* cultures were grown at 37°C and *A. tumefaciens* cultures at 28°C.

Table 3.1 *E. coli* strains used in this study

Strain	Description	Maintenance	Reference
Escherichia coli			
HB101	Highly transformable strain used for large-scale plasmid production	Sm^R	Sambrook *et al.* (1989)
JM83	Suitable strain for bacterial transformations. Exhibits high transformation frequencies	$RecA^-$, Sm^R	Armitage *et al.* (1988)

$RecA^-$ = recombination deficient

Sm^R = streptomycin resistance

Table 3.2 ***A. tumefaciens*** **strains used in this study**

Strain	Description	Maintenance	Reference
Agrobacterium tumefaciens			
Ach5	Wild-type, octopine strain	No selection	Byrne *et al.* (1987)
C58	Wild-type, nopaline strain	No selection	Byrne *et al.* (1987)
C58C1	C58 cured for pTIC58	Rf^R	van Larebecke *et al.* (1974)
C58C1(pGV2260)	C58C1 containing the binary vector pGV2260	Rf^R, Cb^R	Deblaere *et al.* (1987)
C58C1(pMP90)	C58C1 containing the binary vector pMP90	Rf^R, Gm^R	Koncz and Schell (1986)
LBA4404(pAL4404)	Ach5 chromosomal background containing the helper Ti plasmid pAL4404. Non-tumourigenic	Rf^R	Hoekema *et al.* (1983)
EHA101(pEHA101)	Derived from A281. Non-tumourigenic	Rf^R, Km^R	Hood *et al.* (1986b)
EHA105(pEHA105)	Derived from EHA101 by deletion of the kanamycin resistance gene	Rf^R	Liu *et al.* (1992)
C58C1(pEHA101)	C58C1 containing the Ti helper EHA101	Rf^R, Km^R	AgrEvo
C58C1(pATHV)	C58C1 containing the Ti helper ATHV	Rf^R	AgrEvo

Cb^R = carbenicillin resistance
Gm^R = gentamycin resistance
Km^R = kanamycin resistance
Rf^R = rifampicin resistance

Table 3.3 Plasmids used in this study

Plasmid	Description	Maintenance	Reference
pRK2013	Conjugative plasmid used to mobilize binary vectors from *E. coli* to *A. tumefaciens* in a triparental mating	Km^R	Ditta *et al.* (1980)
pBI121	Binary vector plasmid based on pBIN19. Encodes the *uid*A gene (GUS)	Km^R	Clontech Labs. Inc.
pOCA/Ac	Encodes the *pat* gene (Basta resistance). Low copy number plasmid	Tc^R	AgrEvo
pB2/35SAcK	Encodes the *pat* gene (Basta resistance). High copy number plasmid	Ap^R	AgrEvo
pGV2260	A non-oncogenic helper Ti plasmid with T-DNA deleted and replaced with pBR322. Derived from pTiB6S3	Cb^R	Deblaere *et al.* (1987)
pMP90	A non-oncogenic helper Ti plasmid derived from C58	Gm^R	Koncz and Schell (1986)
pAL4404	Derivative of pTiAch5. Contains *vir* region but no T-DNA. Provides *trans*-acting virulence functions in binary systems	Rf^R	Hoekema *et al.* (1983)
pEHA101	Derivative of pTiBo542 but no T-DNA	Km^R	Hood *et al.* (1986a)
pEHA105	Derivative of pEHA101	Cb^R	Liu *et al.* (1992)
pATHV	Ti helper plasmid	No selection	AgrEvo

Table 3.3 Plasmids used in this study (cont.)

Plasmid	Description	Maintenance	Reference
pJIT119	Binary vector plasmid encoding *sul*I (asulam), *uid*A (GUS) and *npt*II (neomycin phosphotransferase). Based on pBIN19	Km^R	Plant Gene Tool Kit Consortium, Norwich, UK
pBI-H	Modified pBI121 containing the hygromycin fragment from pJIT72. Encodes the *uid*A gene (GUS)	Hm^R	Food Science and Technology, CSIR, SA
pHoe6/Ac	Binary vector containing only the *pat* gene in its T-DNA	Sm^R/Sp^R	AgrEvo
pHoe106/Ac	Binary vector containing only the *pat* gene in its T-DNA	Sm^R/Sp^R	AgrEvo
pHoe200/Ac	Binary vector containing only the *pat* gene in its T-DNA	Km^R	AgrEvo
pCNL35	Binary vector containing multiple agropine-type *vir* genes from pTiBo542. The *uid*A gene contains an intron so GUS activity is only expressed in plant cells after the T-DNA has been transferred	Km^R, Ap^R	Liu *et al.* (1992)

Ap^R = ampicillin resistance
Cb^R = carbenicillin resistance
Gm^R = gentamycin resistance
Hm^R = hygromycin resistance
Km^R = kanamycin resistance
Rf^R = rifampicin resistance
Sm^R = streptomycin resistance
Sp^R = spectinomycin resistance
Tc^R = tetracycline resistance

3.2.2 Plasmid DNA extraction

Large scale extractions of pOCA/Ac and pBI121 plasmid DNA from *E. coli* were performed using a modified version of the procedure of Armitage *et al.* (1988). A single colony of the desired strain of *E. coli* was grown in 500 ml of Luria broth (LB) (Appendix B) by shaking at 175 rpm at 37°C overnight. Following centrifugation, 20 ml lysis solution (Appendix B) without lysozyme was added to each pellet in 250 ml centrifuge tubes. These pellets were left to shake in the lysis solution at 100 rpm at 30°C for 30 minutes to resuspend and were then pooled. Lysozyme was added and the suspension was incubated on ice for five minutes to lyse the cells. Instead of adding 100 ml propan-2-ol to the cell suspension and centrifuging for ten minutes as stated in the method of Armitage *et al.* (1988), the centrifuge tube was filled to the brim with propan-2-ol and centrifuged for 20 minutes. The resulting DNA pellet was rinsed in 70% ethanol, air dried and resuspended in 2 ml tris-EDTA buffer (TE) (Appendix B). Nine ml of a cesium chloride stock solution (Appendix B), was added to the DNA along with 400 µl of 10 mg/ml ethidium bromide stock. Samples were processed further according to the method of Armitage *et al.* (1988) but dialysis to remove the cesium chloride was carried out using 1 X TE instead of water and the DNA preparation was stored at 4°C.

Also tested for plasmid DNA extraction were the Promega Wizard Maxiprep System, this kit used in conjunction with the "terrific broth" method (Sambrook *et al.*, 1989) and the chloramphenicol amplification method (Sambrook *et al.*, 1989), as well as this kit modified so that four times the usual number of cells and only one maxicolumn were used to process all four samples. These methods were tested because pOCA/Ac and pBI121 are low copy number plasmids (Armitage *et al.*, 1988; G. Donn, Personal Communication) and it was very difficult to obtain enough DNA to be seen on an agarose gel (at least 30 ng is required per well). It was hoped that these methods would enhance the recovery of the plasmid DNA.

The Promega Wizard Maxiprep System without modifications was used for extraction from *E. coli* of plasmid DNA of high copy number plasmids such as pB2/35SAcK. DNA concentrations were determined by running samples on 1% Seakem agarose gels in

tris-acetate (TAE) buffer (Appendix B) for two hours at 75 volts and comparing the DNA bands with DNA bands of known concentrations.

Small scale DNA extractions were performed using the Rapid Pure Miniprep System obtained from BIO101 but with four times the usual number of cells.

3.2.3 Purification of plasmid DNA

Initially the phenol extraction method of Sambrook *et al.* (1989) was used to remove protein from the DNA preparation. The volume of the DNA solution was increased to at least 100 µl with TE. An equal volume of 1:1 phenol:chloroform was added and mixed. The suspension was centrifuged for ten minutes at room temperature instead of at 4°C for two minutes as stated in the method of Sambrook *et al.* (1989). Phenol equilibration was carried out according to the method of Sambrook *et al.* (1989) but instead of using a magnetic stirrer and aspirator, the phenol and tris-HC1 were mixed by shaking and then poured into a separation funnel so that the phenol layer could be collected. The DNA purification method was later replaced by the Promega DNA Clean-Up System and the BIO101 GeneClean Kit.

3.2.4 Lithium chloride precipitation of plasmid DNA

To concentrate a DNA preparation, the DNA can be precipitated and resuspended in a smaller volume of liquid. The method of Armitage *et al.* (1988) was used but instead of sodium acetate, lithium chloride (LiCl) was used. The volume of the DNA preparation was measured and 4 M LiCl was added to the preparation at a concentration of 10% volume/volume and mixed. One ml of cold (-20°C) 96% ethanol was added and mixed. The preparation was stored at -20°C for at least 15 minutes. Thereafter, centrifugation was carried out at 12 000 rpm for 15 minutes at 4°C. DNA pellets were air dried at room temperature and then resuspended in an appropriate volume of TE.

3.2.5 Mapping of the plasmid pOCA/Ac

Restriction enzyme mapping was used to confirm the structure of the plasmid pOCA/Ac shown in Appendix C. The enzymes used in the digestions were *Pst* I, *Cla* I, *Eco* RI, *Sal* I and *Hind* III (Boehringer Mannheim). H buffer (Boehringer Mannheim) was required in all instances except in the case of *Hind* III where B buffer (Boehringer Mannheim) was optimal for the enzyme. DNA was digested for three hours at 37°C and then loaded into the wells of a 1.5% Seakem agarose gel in tris-borate (TBE) buffer (Appendix B). DNA markers and uncut pOCA/Ac were also loaded. The gel was run at 36 volts overnight, stained with ethidium bromide for 1½ hours and photographed. Only pOCA/Ac was mapped in such detail. To give a rough indication that the plasmid maps available were correct, the plasmids pB2/35SAcK and pBI121 were digested with *Eco* RI to confirm that the number of *Eco* RI restriction sites corresponded with the information given on the maps (Appendix C).

3.2.6 Cloning of the *pat* gene into pBI121

Figure 3.1 shows the strategy used to clone the *pat* gene into pBI121. Maps of pB2/35SAcK and pBI121 are shown in Appendix C. Initially the *pat* gene was isolated from the low copy number plasmid pOCA/Ac. The high copy number plasmid pB2/35SAcK was later obtained from AgrEvo and used in subsequent cloning experiments as the source of the *pat* gene.

Digestions were carried out at 37°C for one hour. Digested DNA of pB2/35SAcK was run on a 2% Seakem agarose gel in TAE buffer (TAE buffer is required for BIO101's GeneClean Kit) for two hours at 75 volts. The 1.329 kb band containing the *pat* gene and 35S promoter and terminator (Appendix C) was identified, cut out of the gel using a scalpel blade and transferred to a sterile eppendorf tube. The band was "glassmilked" (GeneClean Kit, BI0101) to remove the agarose and then ligated with pBI121 cut with *Eco* RI using the method of Sambrook *et al.* (1989). Ligation was carried out overnight.

Two experimental controls were used, namely the vector on its own and the insert on its own.

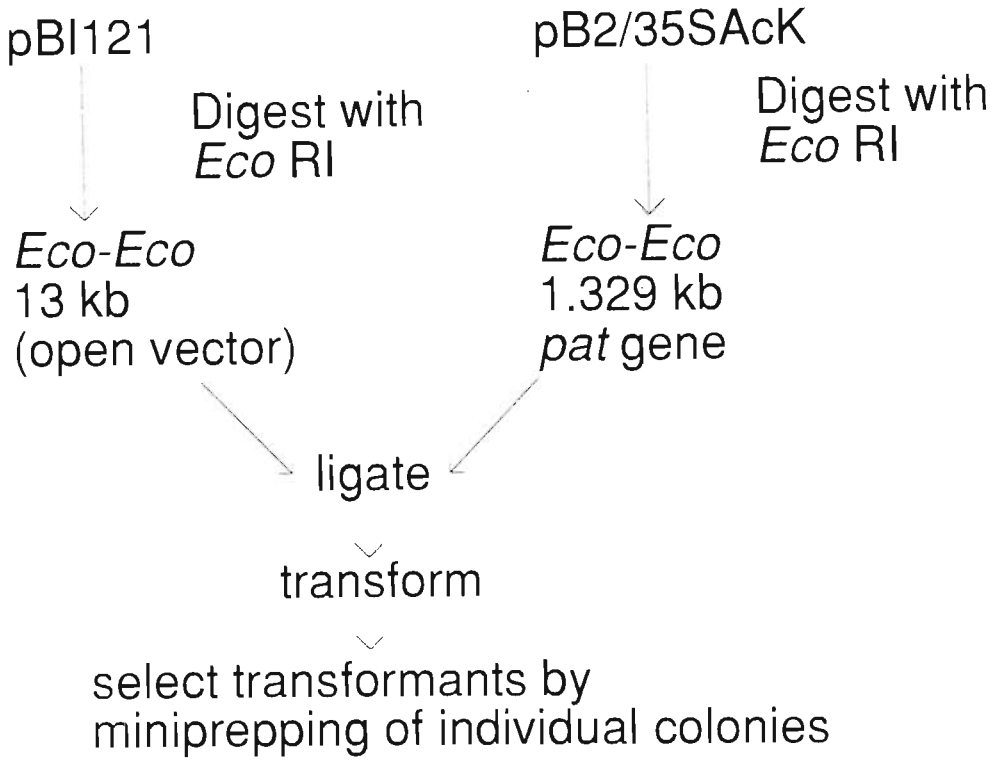

Figure 3.1 Diagram of the cloning strategy used to insert the *pat* gene into the binary vector pBI121

3.2.7 Preparation of competent cells

Two methods of preparing *E. coli* cells which are competent for transformation were compared, namely the method of Armitage *et al.* (1988) and that of Sambrook *et al.* (1989). Test transformations were carried out using DNA dilutions of uncut pBI121 plasmid DNA. For competence, 10^6 colonies/µg DNA are optimal i.e. 100 colonies per LA plate are expected at DNA concentrations of 100 pg/plate, 10 colonies at 10 pg/plate and 1 at 1 pg/plate.

3.2.8 Transformation of competent cells

When competent *E. coli* cells were produced according to the method of Armitage *et al.* (1988), the associated transformation method of Armitage *et al.* (1988) was used. Similarly, when competent *E. coli* cells were produced according to the method of Sambrook *et al.* (1989), the transformation method of Sambrook *et al.* (1989) was used.

3.2.9 Selection of bacterial transformants

Transformants were selected by patching individual *E. coli* colonies onto LA Km_{50} plates. A sample of colonies was miniprepped and digested with *Eco* RI (Boehringer Mannheim). DNA samples were run on a 2% Seakem agarose gel to determine whether any of the colonies contained the vector DNA (pBI121) with the *pat* gene insert.

3.2.10 Conjugation of recombinant plasmids into *A. tumefaciens*

The conjugation method of Armitage *et al.* (1988) was used to transfer the new pBI121 plasmid construct containing the *pat* gene from *E. coli* into the *Agrobacterium* strains LBA4404, C58C1(pMP90), C58C1(pGV2260) and EHA105 via a triparental mating.

3.3 RESULTS

3.3.1 Plasmid DNA extraction

Use of the Promega Wizard Maxiprep Kit without modifications for extracting DNA from high copy number plasmids such as pB2/35SAcK yielded large quantities of DNA (1µg/µl). Maxipreps of low copy number plasmids such as pBI121, prepared using the method of Armitage *et al.* (1988) yielded very little DNA (1µg/300µl). As approximately

10 to 20 µl of DNA can be loaded per well on an agarose gel, if the DNA is at a concentration of 1µg/300µl, this is equivalent to 30 to 60 ng of DNA. This amount of DNA is only just visible on an agarose gel and the yield of DNA using this extraction method was therefore not sufficient for cloning purposes. The Promega Wizard Maxiprep Kit yielded approximately 1µg/100µl of pBI121 DNA which is still not optimal. For high copy number plasmids 1µg/µl of DNA is expected. Use of the "terrific broth" and chloramphenicol amplification methods in DNA maxipreps resulted in large quantities of polysaccharides and very little DNA being isolated. Maxipreps prepared in this manner could not be used for DNA cloning experiments because of the viscous nature of the preparation and the low concentration of DNA. When four times the number of cells were prepared and only one maxicolumn of the Promega kit was used, concentrations of 1µg/10µl of pBI121 DNA could be obtained which was sufficient for cloning purposes. Normally ligation mixes are made up in total volumes of between 10 and 20 µl, and 500 ng DNA of both the insert and the vector are used. The modified Promega maxiprep method therefore yielded adequate low copy number plasmid DNA to perform these ligations.

Minipreps of low copy number plasmids yielded sufficient DNA for digestion and visualisation on gels. DNA concentrations could be improved by resuspending the final pellet in 25 µl TE buffer instead of 50 µl buffer. Mini-dialysis of these DNA preparations was required for effective restriction enzyme digestion. The modified BIO101 miniprep procedure was therefore routinely used to confirm successful cloning.

3.3.2 Purification of plasmid DNA

The Promega DNA Clean-Up System and the BIO101 GeneClean Kit were both more efficient at purifying plasmid DNA, easier to use and less time consuming than the phenol extraction method of Sambrook *et al.* (1989) and were therefore used routinely. The GeneClean Kit was preferred because it did not require the use of a vacuum manifold which was required for the Promega DNA Clean-Up System although better results were obtained with the Promega kit.

3.3.3 Lithium chloride precipitation of plasmid DNA

Lithium chloride precipitation of plasmid DNA was successfully performed. However, the use of the Promega DNA Clean-Up System and the BIO101 GeneClean Kit eliminated the need for lithium chloride preciptation as DNA could be adequately concentrated using these procedures. This was desirable as lithium chloride is toxic.

3.3.4 Mapping of the plasmid pOCA/Ac

Figure 3.2 shows the fragments obtained from the digestion of pOCA/Ac with various restriction enzymes. The size of the fragments obtained was determined by drawing a standard curve of the distance moved by marker bands during electrophoresis versus the log of the size of the bands. This resulted in a best-fit curve. The distance moved by each of the plasmid fragments during electrophoresis was measured and a corresponding fragment size estimated from the standard curve. Using this method, it was found that the size of the fragments of pOCA/Ac corresponded to those given in the plasmid map obtained from AgrEvo (Appendix C).

3.3.5 Cloning of the *pat* gene into pBI121

Figure 3.3 shows the products obtained from the digestion of pBI121 and pB2/35SAcK with *Eco* RI (Boehringer Mannheim). The 1.329 kb band, *pat*, from pB235S/AcK, was removed from the agarose gel using the GeneClean Kit (BI0101) and ligated with pBI121 cut with *Eco* RI. Figure 3.4 shows the products of this ligation and of the control ligations.

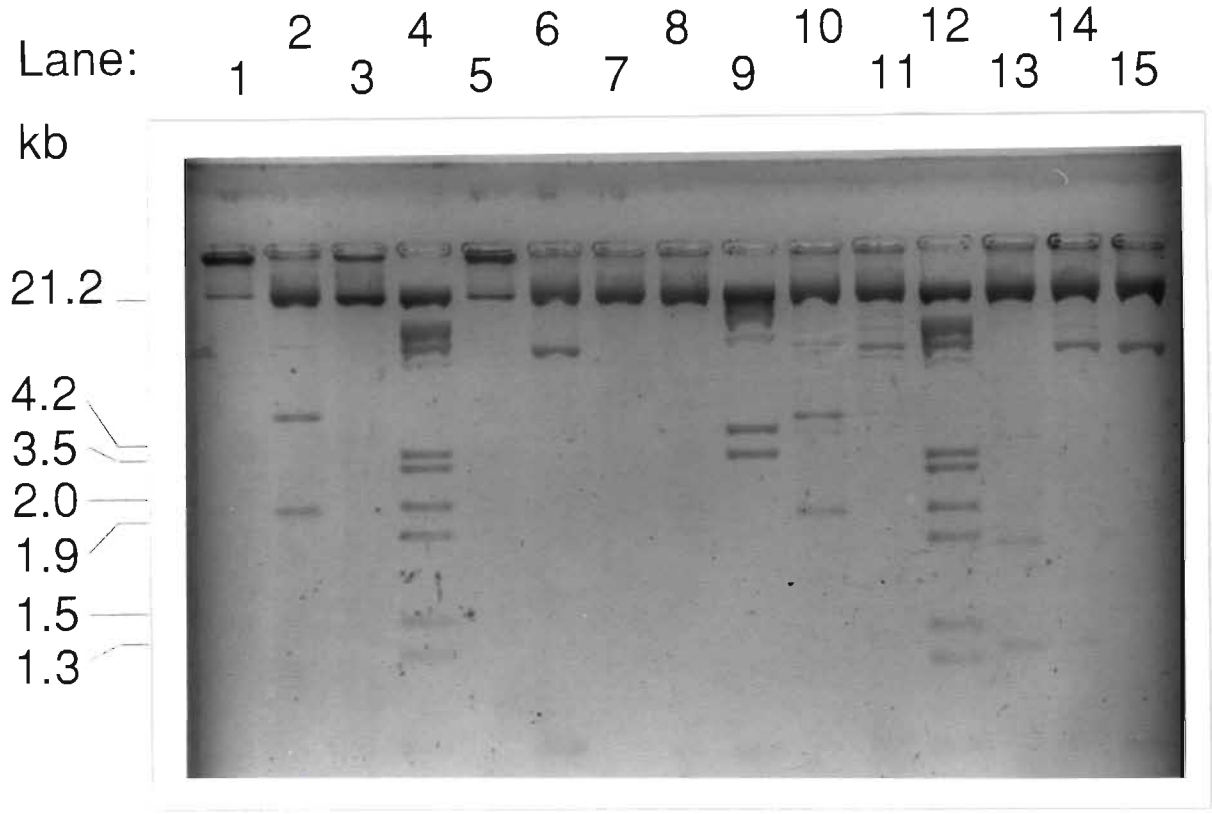

Figure 3.2 Agarose gel showing the digestion of pOCA/Ac with a number of restriction enzymes (All enzymes and markers obtained from Boehringer Mannheim) Lane 1: Uncut pOCA/Ac; Lane 2: *Pst* I digestion; Lane 3: *Cla* I digestion; Lane 4: λ III marker; Lane 5: *Eco* RI digestion; Lane 6: *Sal* I digestion; Lane 7: *Hin*d III digestion; Lane 8: *Pst* I/Cla I digestion; Lane 9: λ II marker; Lane 10: *Pst* I/*Eco* RI digestion; Lane 11: *Pst* I/*Sal* I digestion; Lane 12: λ III Marker; Lane 13; *Cla* I/*Eco* RI digestion; Lane 14: *Cla* I/*Sal* I digestion; Lane 15: *Eco* RI/*Sal* I digestion

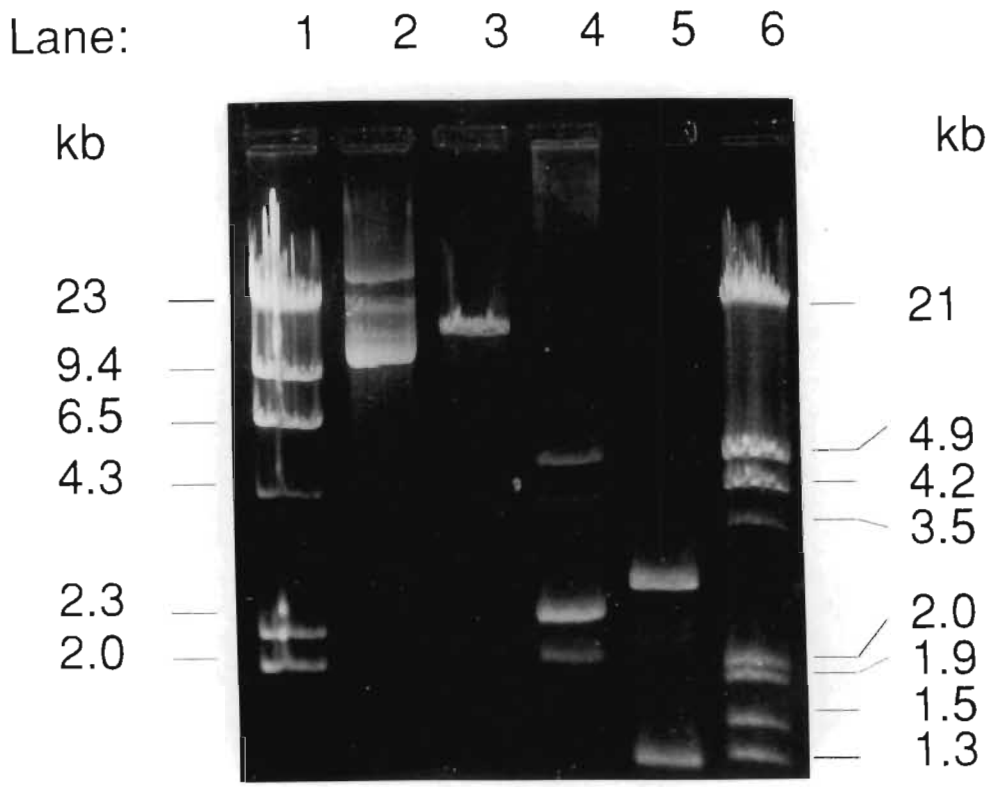

Figure 3.3 Agarose gel showing the digestion of pBI121 and pB2/35SAcK with the restriction enzyme *Eco* RI (all enzymes and markers obtained from Boehringer Mannheim). Lane 1: λ II marker; Lane 2: uncut pBI121; Lane 3: pBI121 cut with *Eco* RI; Lane 4: uncut pB2/35SAcK; Lane 5: pB2/35SAcK cut with *Eco* RI; Lane 6: λ III marker

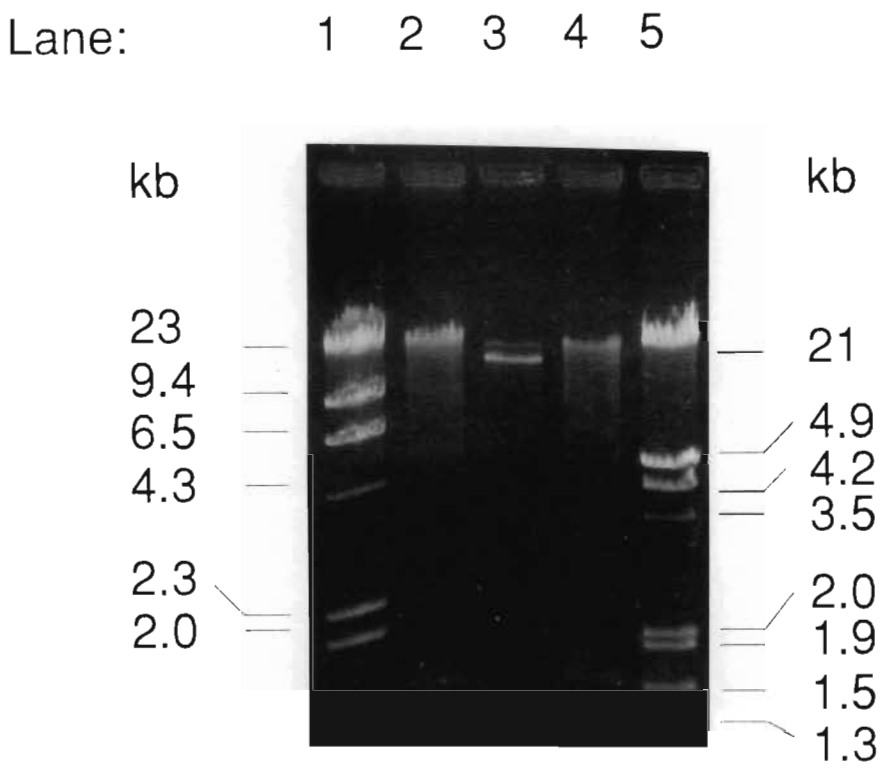

Figure 3.4 **Agarose gel showing the ligation of the *pat* gene into pBI121 cut with the restriction enzyme *Eco* RI (all enzymes and markers obtained from Boehringer Mannheim). Lane 1: λ II marker; Lane 2: pBI121 cut with *Eco* RI and ligated with the *pat* gene; Lane 3: pBI121 cut with *Eco* RI and ligated; Lane 4: the *pat* gene ligated; Lane 5: λ III marker**

3.3.6 Preparation of competent cells

The method of Sambrook *et al.* (1989) for preparing competent cells was superior to that of Armitage *et al.* (1988) although not even the former produced the 10^6 colonies per µg of DNA required to confirm that cells were competent for transformation. At least 10 colonies of the 100 pg concentration of uncut pBI121 DNA were required per plate to ensure competence (Table 3.4).

Table 3.4 Determination of *E. coli* strains competent for transformation

Concentration of uncut pBI121 DNA (pg)	Number of colonies per plate	
	Method of Armitage *et al.* (1988)	Method of Sambrook *et al.* (1989)
100	0	32
10	0	2
1	0	0

Further experiments using the method of Sambrook *et al.* (1989) but employing centrifuge tubes instead of eppendorf tubes in the transformation procedure were conducted. This improved the frequency of transformation by allowing better expression of the antibiotic resistance genes and resulted in a greater number of colonies being produced (Table 3.5).

Table 3.5 Determination of *E. coli* strains competent for transformation using a modified transformation procedure

Concentration of uncut pBI121 DNA (pg)	Number of colonies per plate
100	96
10	4
1	0

3.3.7 Transformation of competent cells

Although the modified Sambrook *et al.* (1989) method still produced low transformation frequencies in the test transformation using known DNA concentrations (96 colonies instead of 100 were obtained), transformation was nevertheless attempted. Table 3.6 shows the results of this experiment.

Table 3.6 Transformation of the *E. coli* strain JM83 with pBI121 containing a *pat* gene insert[a]

Ligation mix	Colony counts on LA Km_{50} plates
Vector and insert (pBI121 cut with *Eco* RI, and *pat*)	117
Vector (pBI121 cut with *Eco* RI)	18
Insert (*pat*)	0

a: kanamycin was used to select for transformants

3.3.8 Selection of bacterial transformants

Figure 3.5 shows the products of restriction enzyme digestion of DNA extracted from a sample of colonies obtained from the transformation of the vector and insert ligation mix (pBI121 cut with *Eco* RI, and *pat*) into competent *E. coli* cells.

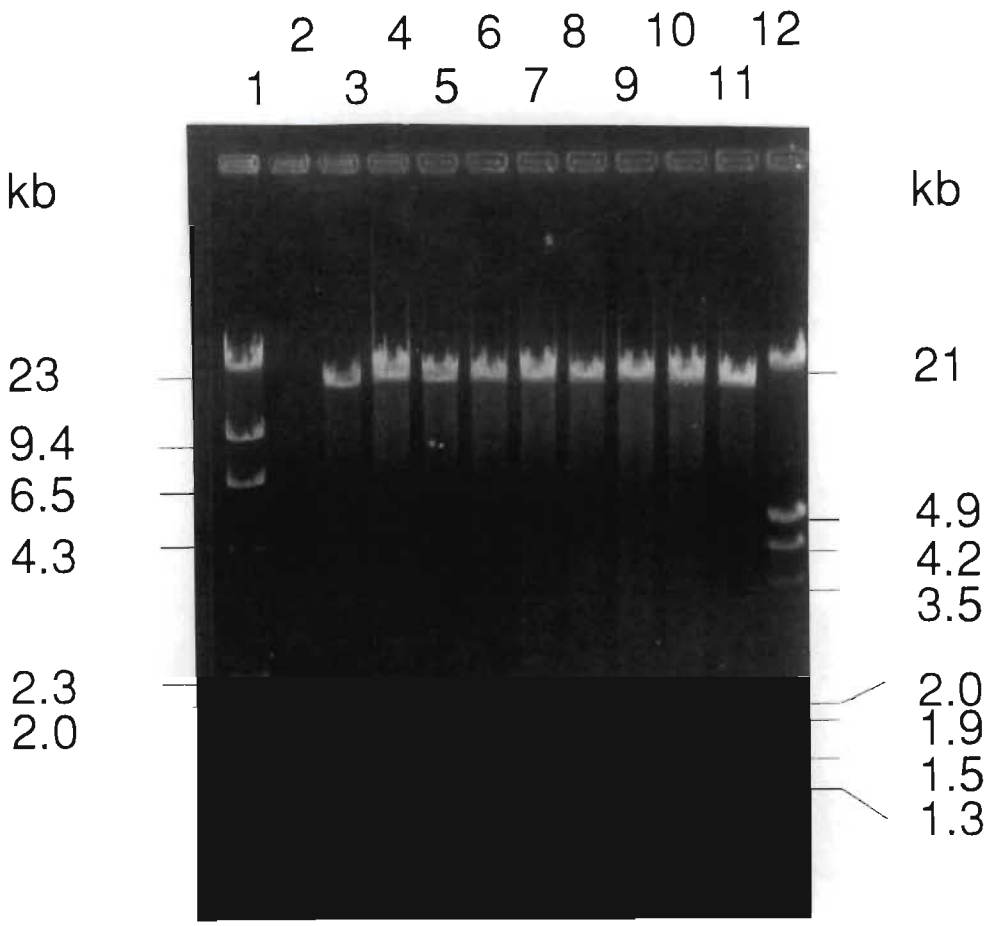

Figure 3.5 **Agarose gel showing the digestion of DNA isolated from transformed *E. coli* colonies containing the vector pBI121 and a *pat* gene insert (all enzymes and markers obtained from Boehringer Mannheim). Lane 1: λ II marker; Lane 2: the *pat* gene; Lane 3: pBI121 cut with *Eco* RI; Lanes 4-8: colonies containing the *pat* gene; Lanes 9-11: colonies without the *pat* gene; Lane 12: λ III marker**

3.3.9 Conjugation of recombinant plasmids into *A. tumefaciens*

The method of Armitage *et al.* (1988) was successfully used to transfer the pBI121 plasmid containing the *pat* gene (new construct called pBI121/Ac) into the disarmed *Agrobacterium* strains LBA4404, C58C1(pMP90), C58C1(pGV2260) and EHA105. The

confirmation that pBI121/Ac was successfully conjugated into the *Agrobacterium* strains was based only on the ability of the strains to grow on medium containing antibiotics which selected for the plasmid. DNA analysis was not conducted to confirm the presence of pBI121/Ac in the *Agrobacterium* strains.

3.4 DISCUSSION

With the introduction of molecular biology kits onto the market, gene manipulation has become much simpler. It is now possible to extract large quantities of DNA from low copy number plasmids where previously these plasmids were almost impossible to isolate. Even so, these kits required modification for use with low copy number plasmids. In this study, a modification of the Promega Wizard Maxiprep System was routinely used for large scale DNA extraction from low copy number plasmids. The kit without modifications was used for the extraction of DNA from high copy number plasmids. The Rapid Pure Miniprep System (BI0101) was routinely used for small scale plasmid DNA extractions and modified for use with low copy number plasmids.

Also routine was the use of the Promega DNA-Clean Up system to purify and concentrate DNA obtained from maxipreps. Mini-dialysis was sufficient for purification of DNA obtained from minipreps. The GeneClean Kit (BI0101) was routinely used to remove agarose from DNA following electrophoresis. The method of Sambrook *et al.* (1989) for the preparation and transformation of competent cells was preferred to that of Armitage *et al.* (1988) because it resulted in a larger number of transformants.

The *pat* gene was successfully cloned into the binary vector pBI121 which contains the GUS reporter gene, *uid*A. The new construct was named pBI121/Ac and was conjugated into four disarmed *Agrobacterium* strains for use in plant transformation experiments. It is useful to have the GUS marker gene for early detection of plant transformation and the *pat* gene for later selection but it must be noted that this construct will only be used for research purposes to establish plant transformation

systems. Constructs containing only the *pat* gene are preferable for the production of transgenic Basta resistant plants for commercial purposes.

CHAPTER 4

DEVELOPMENT OF TRANSFORMATION SYSTEMS FOR THE TRANSFER OF FOREIGN GENES TO SOUTH AFRICAN SOYA BEAN CULTIVARS

4.1 INTRODUCTION

Conventional plant breeding techniques have been widely used to improve soya bean seed yield, available protein and oils and resistance to microbes and other pests. However, with the development of plant regeneration systems, soya bean can be improved by the use of tissue culture for the selection of desirable traits (mutations, somaclonal variation) or by using tissue culture to aid the transfer of genetic information. Either *Agrobacterium*-mediated transformation or direct DNA transfer methods can be applied to soya bean (Chee *et al.*, 1989). The transfer of foreign genes into agronomically important crops depends upon the efficient transfer of genes into cells capable of regenerating into fertile plants (Dhir *et al.*, 1991a). The genetic improvement of soya bean has been limited due to the lack of an efficient transformation system and the inability to regenerate plants from transformed cells or tissues (Facciotti *et al.*, 1985; Christou *et al.*, 1990).

Soya bean is a dicotyledonous plant and is therefore potentially susceptible to *Agrobacterium* infection. Over the years, a number of groups (Pedersen *et al.*, 1983; Owens and Cress, 1985; Hood *et al.*, 1986a; Kudirka *et al.*, 1986; Byrne *et al.*, 1987; Hood *et al.*, 1987; Owens and Smigocki, 1988) have studied the response of soya bean to *Agrobacterium* infection and have established that soya bean can be infected by *Agrobacterium*. Infectivity tests however, show that not all *Agrobacterium* strains can infect soya bean tissues and that the nopaline-type strains which code for the synthesis

of the novel amino acid nopaline are the most effective for soya bean infection (Widholm, 1988; Chee *et al.*, 1989).

Foreign genes were first introduced into soya bean tissues by Facciotti *et al.* (1985). Kanamycin resistant callus was obtained using an *Agrobacterium*-mediated transformation method but no transgenic plants were obtained. It was only in 1988 that successful production of genetically modified soya bean plants occurred. This was achieved by two different research groups using different methods. Hinchee *et al.* (1988) of Monsanto used *A. tumefaciens* to transform shoots which arose via organogenesis from cotyledons. The cotyledons were removed from germinating seedlings of soya bean genotypes previously found to be susceptible to *A. tumefaciens* infection. Kanamycin and glyphosate resistant plants were obtained but at low frequencies (6%). Christou *et al.* (1988) and McCabe *et al.* (1988) of Agracetus used a microprojectile system in which DNA-coated gold particles were accelerated into meristems of embryonic axes from immature soya bean seeds. Approximately 2% of the shoots produced by organogenesis were chimaeric for the β-glucuronidase or *npt*II genes. One of the plants transformed with the *npt*II gene produced some transformed progeny, indicating that self-fertilization of flowers in the transformed portions of the chimaeric plants can produce wholly transformed plants.

Various other groups have had some success with soya bean transformation. Chee *et al.* (1989) used *A. tumefaciens* to infect germinating seeds of soya bean but the yield of transgenic plants was extremely low (0.07%). Chimaeric plants which produced no transgenic progeny were obtained by Parrott *et al.* (1989) using *A. tumefaciens* to infect somatic embryos on cotyledons of soya bean. Zhou and Atherly (1990) used a method based on that of Hinchee *et al.* (1988) and produced transgenic soya bean calli. Routine transformation via particle bombardment of embryogenic suspension culture tissue of soya bean was achieved by Finer and McMullen (1991). Dhir *et al.* (1992b) reported the production of transgenic soya bean plants from electroporated protoplasts but this was later retracted (Dhir *et al.*, 1993). Recently, *Agrobacterium* was used to transform cotyledonary nodes of soya bean (Townsend and Thomas, 1994).

Some work on *in vitro* genetic manipulation of soya bean has been conducted by South African researchers. McKenzie and Cress (1992) evaluated South African soya bean cultivars for their susceptibility to *A. tumefaciens* and obtained transgenic plants of the cultivar Forrest. Austin and Cress (1994) detected tumours produced in response to infection with *A. tumefaciens* in twelve South African soya bean cultivars.

Because of the problems associated with *Agrobacterium* host specificity, differences in the regenerability of cultivars and the difficulties of obtaining transformation and regeneration events in the same cell types, *Agrobacterium*-mediated transformation is not the method of choice to transform soya bean. Direct DNA transfer methods are very useful in genetic engineering of soya bean (Chee *et al.*, 1989; Christou *et al.*, 1990) because they overcome problems such as the genotype specificity associated with *Agrobacterium*-mediated transformation. It is important to note that at present the direct DNA transfer method of Agracetus, known as the Accell Gene Delivery System, first reported by Christou *et al.* (1988) and McCabe *et al.* (1988) is the only method in commercial use for the genetic engineering of soya bean in a variety-independent fashion. Field trials of herbicide resistant soya bean (Round-Up using the *aro*A gene and Basta using the *bar* gene, not the *pat* gene), produced using the Agracetus particle gun method, have been carried out over the last three years with expected commercialization in the very near future (Christou, 1994).

This chapter focuses on *Agrobacterium*-mediated transformation to transform soya bean. The use of the particle gun was avoided because this method was patented by Agracetus and involves the payment of royalties and fees. It must be noted, however, that although some success using *A. tumefaciens* has been achieved by Hinchee *et al.* (1988), Chee *et al.* (1989), Parrott *et al.* (1989), Zhou and Atherly (1990) and Townsend and Thomas (1994), most of the current research on the genetic engineering of soya bean involves microprojectile systems (Finer and McMullen, 1991; Christou, 1994). Also included in this chapter is the transformation of the non-recalcitrant crop, tobacco, which is a model system for plant genetic engineering.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and plasmids

Bacteria and plasmids used in transformation experiments are shown in Tables 3.1, 3.2 and 3.3 in Chapter 3. Maps of the plasmids pHoe6/Ac, pHoe106/Ac, pHoe200/Ac, pBI121 and pJIT119 are given in Appendix C. All Agrobacteria were maintained on Luria agar (LA) (Appendix B) containing the relevant antibiotics and grown at 28°C. Transconjugant strains were obtained using the conjugation method of Armitage *et al.* (1988) to transfer binary vector plasmids into *Agrobacterium* strains via triparental matings. Cultures of *Agrobacterium* for use in transformation experiments were obtained by inoculating a single colony of the desired strain into Luria broth (LB) (Appendix B) and shaking at 175 rpm for 48 hours at 28°C. Aliquots of the culture were washed by centrifugation at 11 000 rpm for three minutes to remove traces of the antibiotics. The pellets were resuspended in sterile distilled water. Unless otherwise stated, cultures were diluted to an OD_{550} of approximately 0.35. Dilutions of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} were plated out onto LA plates containing selective antibiotics. This step was carried out to determine whether the bacterial concentration was between 5×10^{8} and 5×10^{10} colonies/ml which is optimal for the transformation of soya bean (Townsend and Thomas, 1994).

4.2.2 Plant material and culture media

Cell suspension cultures, embryonic axes, hypocotyls and cotyledons of the soya bean cultivars Forrest, Hutton and PNR577G were used in transformation experiments. Plant tissue culture media used in these experiments were the same as the regeneration media described in Chapter 2 and Appendix A but contained cefotaxime (Cx) and either kanamycin (Km) or glufosinate ammonium (Glu Am) depending on the binary vector used. The minimum inhibitory concentrations of the chemical agents used to select for transformed soya bean and tobacco plant material are summarized in Tables A and B

in Appendix D. The reagents for the β-glucuronidase (GUS) histochemical assay of Jefferson (1987) are given in Appendix D. After the assay, plant samples were cleared of chlorophyll using formyl-aceto-alcohol (Appendix D) in order to make the blue colour easier to visualize in GUS positive samples.

4.2.3 Use of acetosyringone to induce virulence genes

Acetosyringone has been shown to increase *Agrobacterium* strain virulence on soya bean (Owens and Smigocki, 1988; Godwin *et al.*, 1991). The compound was therefore added routinely to *Agrobacterium* cultures and to the tissue culture media used for co-cultivation, at a concentration of 0.5 mg/l.

4.2.4 Determination of concentrations of cefotaxime that controlled the growth of *A. tumefaciens* without affecting the growth of different soya bean explants

Hypocotyls from untransformed soya bean seedlings of the cultivar Forrest germinated for ten days and cotyledons and embryonic axes from untransformed Forrest seeds germinated for two days were placed on regeneration medium containing 0, 250, 500 or 1000 mg/l cefotaxime (Cx) at 25°C in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$). Fifty hypocotyls, 25 embryonic axes and 50 cotyledons were used to test each cefotaxime concentration. Explants were transferred to fresh medium containing Cx after three days. This step was repeated after a further three days and again three days later to ensure that Cx was always present in the medium as Cx degrades in the light. Thereafter explants were transferred to fresh medium once every seven days for three weeks. The effect of cefotaxime on the soya bean explants was recorded after four weeks.

4.2.5 Determination of concentrations of kanamycin that inhibited the growth of different untransformed soya bean explants

Hypocotyls from untransformed soya bean seedlings of the cultivar Forrest germinated for ten days and cotyledons and embryonic axes from untransformed Forrest seeds germinated for two days were placed on regeneration medium containing 0, 25, 50, 100, 200 or 300 mg/l kanamycin (Km). Fifty hypocotyls, 25 embryonic axes and 50 cotyledons were used to test each kanamycin concentration. Explants were incubated at 25°C in the light for four weeks (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$). The minimum inhibitory concentration (MIC) for kanamycin was found by determining the minimum concentration at which callus was not produced on untransformed hypocotyls and at which shoots were not produced on untransformed embryonic axes and cotyledons.

4.2.6 Determination of concentrations of glufosinate ammonium that inhibited the growth of different untransformed soya bean explants

Hypocotyls from untransformed soya bean seedlings of the cultivar Forrest germinated for ten days were placed on regeneration medium containing 0, 1, 3, 5 or 20 mg/l glufosinate ammonium. Forty hypocotyls were used to test each glufosinate ammonium concentration. After four weeks of culture, the concentration at which the development of callus on the hypocotyls was inhibited was determined. Cotyledons from untransformed soya bean seeds of the cultivar Forrest germinated for two days were placed on regeneration medium containing 0, 1, 3, 5, 10, 20, 40, 60, 80 or 100 mg/l glufosinate ammonium. Sixty cotyledons were used to test each glufosinate ammonium concentration. Routine procedures for regeneration from cotyledons were carried out and after four weeks of culture, the MIC for glufosinate ammonium was found by determining the minimum concentration at which shoots turned brown and died. The MIC of glufosinate ammonium was also determined for the rooting of untransformed soya bean shoots. Concentrations of 0, 1, 3, 5 and 20 mg/l glufosinate ammonium were added to solid ½MS 30 rooting medium. Twenty shoots were used per glufosinate ammonium concentration. Procedures for rooting of soya bean shoots obtained from

cotyledons were carried out. After four weeks, the MIC was found by determining the minimum concentration of glufosinate ammonium at which untransformed shoots were prevented from rooting. The concentration of glufosinate ammonium at which shoots turned brown and died was also recorded.

4.2.7 Preliminary experiments to determine which *A. tumefaciens* strains transform soya bean hypocotyls

The virulent *A. tumefaciens* strains Ach5 and C58 and the avirulent binary vector containing strains C58C1(pGV2260)(pJIT119), LBA4404(pJIT119) and EHA101(pBI-H) were used to infect wounded hypocotyls on seedlings of the cultivars Forrest and Hutton germinated for 14 days. The binary vectors contained the *uid*A gene for β-glucuronidase activity. Each *Agrobacterium* strain was grown for 48 hours in Luria broth (LB) (Appendix B) containing selective antibiotics and 0.5 mg/l acetosyringone. A 1 in 50 dilution (OD_{550} and plate count not determined in this case) of each culture was drawn up into a syringe and the syringe needle used to scratch the surface of each hypocotyl letting two drops of the culture fall onto the wound site. Ten seedlings of each cultivar were used per *Agrobacterium* strain. The wounded seedlings were incubated for four weeks at 25°C in the light (16 hour photoperiod, 37.5 $\mu E.m^{-2}.s^{-1}$) and then examined for tumour formation or assayed for β-glucuronidase (GUS) activity using the histochemical method of Jefferson (1987).

4.2.8 Transformation of cell suspension cultures of soya bean

Cell suspension cultures of soya bean were used in transformation studies because transformation of cell cultures is relatively easily achieved in comparison with the transformation of other soya bean explants (Finer and McMullen, 1991). Ten cell cultures derived from hypocotyl callus cultures of the soya bean cultivar Forrest were inoculated with 100 µl full strength *Agrobacterium* culture per 20 ml suspension culture. The *A. tumefaciens* strain C58C1(pGV2260)(pJIT119) was used. The inoculum was

prepared by growing the culture for 48 hours in LB containing selective antibiotics and 0.5 mg/l acetosyringone. After three days of co-cultivation with C58C1(pGV2260)(pJIT119) at 25°C in the dark, the plant cells were allowed to settle, the liquid MSC 20 medium was removed and fresh medium containing 500 mg/l Cx was added. This step was repeated after a further three days of culture. Cells were plated out onto ten plates of solid MSC 20 medium containing 100 mg/l Km (Table A, Appendix D) and 500 mg/l Cx nine days after inoculation to select transformed microcalli. A sample of cells was also assayed for GUS activity according to Jefferson (1987). Microcalli that grew on the selection medium were assayed for GUS activity 21 days after the cells had been transferred to solid medium.

4.2.9 Transformation of embryonic axes of soya bean

Because shoots can be produced from soya bean embryonic axes, these explants were used in transformation studies. Embryonic axes excised from seeds of the soya bean cultivar Forrest germinated for two days were wounded and inoculated with *Agrobacterium* in various ways. Embryonic axes were either injected with *Agrobacterium* using a needle and syringe, or scratched with a needle or cut with a scalpel blade on the surface and then co-cultivated with *Agrobacterium* for two days at 25°C in the light. Twenty embryonic axes were used to test each variable. A 1 in 50 dilution of cultures of the *A. tumefaciens* strains C58C1(pGV2260)(pJIT119) and C58C1(pMP90)(pJIT119) was used. The inoculum was prepared by growing the culture for 48 hours in LB containing selective antibiotics and 0.5 mg/l acetosyringone. Approximately 20 µl of the 1 in 50 dilution were applied per explant. Following co-cultivation, embryonic axes were placed on selection medium containing 100 mg/l kanamycin (Table A, Appendix D) and 500 mg/l Cx and were assayed for GUS activity one week, two weeks and four weeks after inoculation according to the histochemical method of Jefferson (1987).

4.2.10 Transformation of hypocotyls of soya bean

Although plants could not be regenerated from hypocotyls using any of the methods tested in this study, callus can be obtained from hypocotyls. The transformation of soya bean hypocotyls is more straightforward than the transformation of soya bean cotyledons (Hinchee *et al.*, 1988). It is therefore useful to transform hypocotyls with disarmed *Agrobacterium* strains to determine which strains are able to infect soya bean explants and also to test new constructs. Soya bean hypocotyls were excised from seeds germinated for seven days and cut into 1 cm lengths. The cut ends of 700 hypocotyls were inoculated with 5 µl of the *Agrobacterium* strain C58C1(pGV2260)(pBI121/Ac) diluted to an OD_{550} of approximately 0.35 (1 in 50 dilution). In these experiments, only the *Agrobacterium* strain C58C1(pGV2260)(pBI121/Ac) was tested because C58C1(pGV2260) was previously found to transform soya bean cell suspension cultures (section 4.2.8) and would thus be expected to transform soya bean hypocotyls. The hypocotyl segments were co-cultivated with the *Agrobacterium* inoculum on MSC 20 medium in the light at 25°C for two days. Thereafter, the hypocotyl segments were transferred to MSC 20 medium containing 500 mg/l cefotaxime. Regular transfers to fresh medium were carried out every three days.

After three weeks, the selection agents kanamycin (20 mg/l) and glufosinate-ammonium (1 mg/l) (Table A, Appendix D) were also included in the medium. Late selection was applied because hypocotyls are very sensitive to *Agrobacterium* infection and require some time to recover from bacterial infection if death of the hypocotyl is to be avoided. This observation was based on preliminary experiments where early selection was applied and resulted in the death of the hypocotyls (data not shown). The concentrations of kanamycin and glufosinate ammonium used in the tissue culture medium were lower than the minimum inhibitory concentrations determined previously in sections 4.2.5 and 4.2.6. This is because these two selection agents in combination inhibited the development of callus from hypocotyls more severely than when only one selection agent was used. Again, this was based on experiments in which the use of 100 mg/l kanamycin in conjunction with 5 mg/l glufosinate ammonium (MIC's

determined in sections 4.2.5 and 4.2.6) resulted in the death of hypocotyls (data not shown).

Also tested was the use of a 1 in 100 000 dilution of the *Agrobacterium* culture and a seven day co-cultivation period. The effect of a long co-cultivation period was examined because longer co-cultivation times such as 168 hours instead of the usual 48 hours reduce the effects of *Agrobacterium* strain specificity in plants (G. Donn, Personal Communication). Putatively transformed callus was assayed for GUS activity (Jefferson, 1987) 14, 21 and 28 days after inoculation.

4.2.11 Transformation of cotyledons of soya bean

Fertile plants can be regenerated from cotyledons of soya bean and this factor made these explants the focus of transformation experiments.

4.2.11.1 Wounding of soya bean cotyledons for *A. tumefaciens* infection

One essential aspect in the development of an *Agrobacterium*-based transformation system is the wounding process. In the case of sunflower, reduction in the frequency of regeneration was an indication of the success of the wounding method (S. Hearn, Personal Communication). Wounding experiments on soya bean cotyledons were therefore conducted and the frequency of regeneration assessed.

Cotyledons of the soya bean cultivar Forrest, excised from seeds germinated for two days, were wounded by cutting the cotyledons in half transversally, or shaking for 30 or 60 seconds in 20 ml sterile distilled water containing 5 g beach sand or 100 mg powdered glass. In these experiments, 40 cotyledons were used to test each variable. By allowing cotyledons to dry out in a laminar flow for one hour, the effect of desiccation on regeneration and transformation was also determined. Vain *et al.* (1993) used an

osmotic treatment, a form of desiccation to enhance transformation of maize. The osmotic treatment prevented extrusion of the protoplasm from wounded cells. It was hoped that desiccation would also enhance transformation in soya bean. In these experiments, 120 cotyledons were used to test each variable.

4.2.11.2 Transformation of soya bean cotyledons using *A. tumefaciens*

Various transformation experiments have been conducted in this study using soya bean cotyledons excised from seeds germinated for two days. In initial experiments, cotyledons of the cultivars Forrest, Hutton and PNR577G were wounded by either cutting them in half, scratching their surface with a needle or a scalpel blade, vortexing them in sterile distilled water containing 5 g beach sand for two minutes, or wounding them using a specialized miniature wire brush developed in this lab (termed a "gene brush") prior to co-cultivation with *Agrobacterium*. The strains C58C1(pGV2260)(pJIT119) and C58C1(pMP90)(pJIT119) were used. Sixty cotyledons of each cultivar were used to test each variable. The *Agrobacterium* strains were grown for 48 hours in LB containing selective antibiotics and 0.5 mg/l acetosyringone. A 1 in 50 dilution of the *Agrobacterium* culture was used for the inoculation of cotyledons. Cotyledons were transferred regularly to regeneration medium containing 300 mg/l kanamycin (Table A, Appendix D) and 500 mg/l cefotaxime. A sample of cotyledons was assayed for GUS activity one week, two weeks and four weeks after inoculation, according to the histochemical method of Jefferson (1987).

In further expermiments, a modified feeder layer system was tested. According to Fillatti *et al.* (1987) and J. Fillatti (Personal Communication), an alternative to using a tobacco cell suspension overlaid with filter paper was to use the filter paper on its own placed on the surface of the tissue culture medium. This improved the frequency of tomato cotyledon transformation. The use of filter paper instead of feeder layers was tested using cotyledons of the soya bean cultivars Forrest and Hutton. The *Agrobacterium* strains C58CI(pMP90)(pHoe6/Ac), C58CI(pEHA101)(pHoe106/Ac), C58CI(pATHV)(pHoe200/Ac) and EHA105(pEHA105)(pCNL35) were used in these

experiments. *Agrobacterium* strains were grown at 28°C for 48 hours in LB containing selective antibiotics and 0.5 mg/l acetosyringone. Cultures were diluted to an OD_{550} of approximately 0.35 (1 in 50 dilution) for use as the inoculum in transformation experiments.

Soya bean cotyledons of the cultivars Forrest and Hutton were excised from seeds germinated for two days. Cotyledons were wounded by shaking them vigorously for 30 seconds in 20 ml of sterile distilled water containing either 5 g beach sand or 100 mg powdered glass (ground Pasteur pipettes). In these experiments, 120 cotyledons of each cultivar were used per *Agrobacterium* strain. Prior to inoculation with *Agrobacterium*, cotyledons were cultured in the dark on a piece of sterile filter paper on the surface of regeneration medium. The tissue culture medium was at a pH of 5.5 and cotyledons were cultured at 20°C. Medium with a low pH and low incubation temperatures improved the frequency of soya bean transformation according to Townsend and Thomas (1994).

After two days, cotyledons were inoculated with the diluted cultures (OD_{550} = 0.35) of *Agrobacterium* by soaking in a "bath" of the culture for one hour. Excess liquid was removed by blotting the cotyledons on sterile filter paper. Cotyledons were transferred back to the filter paper on regeration medium for two days in the dark at 20°C before transfer to regeneration medium containing 500 mg/l Cx (without filter paper). The transfer to medium containing Cx was repeated after three days and again after a further three days to eliminate the *Agrobacterium* inoculum. Thereafter, cotyledons were transferred to fresh medium containing Cx once every seven days.

Four weeks after inoculation, cotyledons were transferred to regeneration medium containing 80 mg/l glufosinate ammonium (Table A, Appendix D) for selection of glufosinate ammonium resistant transformed shoots. In the case of cotyledons infected with the strain EHA105(pEHA105)(pCNL35), cotyledons were placed on regeneration medium containing 300 mg/l kanamycin (Table A, Appendix D) to select for kanamycin resistant shoots. These cotyledons and the shoots produced therefrom were assayed for GUS activity according to Jefferson (1987).

The transconjugant strain C58C1(pGV2260)(pBI121/Ac) was also tested in transformation experiments using soya bean cotyledons of the cultivars Forrest and Hutton. Cotyledons were excised from seeds germinated for two days. Explants were wounded by shaking vigorously for two minutes in 20 ml sterile distilled water containing 5 g beach sand. Cotyledons were cultured on regeneration medium for two days at 20°C prior to inoculation with *Agrobacterium*. Either a 1 in 100 000 dilution (OD_{550} not determined) with a seven day co-cultivation period, or a 1 in 50 dilution (OD_{550} = ± 0.35) with a two day co-cultivation period was used. In these experiments, 240 cotyledons of each cultivar were tested per variable. Cotyledons were transferred regularly to fresh medium containing 500 mg/l Cx. After four weeks regenerated shoots were removed from the cotyledons and cultured on rooting medium containing 500 mg/l Cx, 20 mg/l kanamycin and 5 mg/l glufosinate ammonium (Table A, Appendix D) . A sample of shoots was assayed for GUS activity (Jefferson, 1987).

4.2.12 Transformation of tobacco, a model system for plant genetic engineering, with *A. tumefaciens* strains containing the *pat* gene or the *pat* gene and the *uid*A gene

Due to the difficulties associated with soya bean transformation, a model system for plant genetic engineering was used to test *Agrobacterium* strains containing the *pat* gene and to obtain Basta resistant material with which to optimize assay systems (section 4.2.13). Transgenic tobacco plants of the South African cultivar A4 were regenerated on MSS 30 medium (Appendix A) using the leaf disc method of Horsch *et al.* (1985). A 1 in 50 dilution (OD_{550} = 0.35) of each of the *Agrobacterium* strains C58C1(pMP90)(pHoe6/Ac), C58C1(pEHA101)(pHoe106/Ac), C58C1(pATHV)(pHoe200/Ac), C58C1(pMP90)(pBI121/Ac), C58C1(pGV2260)(pBI121/Ac), LBA4404(pBI121/Ac) and EHA105(pBI121/Ac) was used to infect tobacco leaf discs. Fifty leaf discs were used per strain except in the case of C58C1(pMP90)(pBI121/Ac) where only 15 leaf discs were used.

The selection agents used to select for transformed tobacco material are given in Table B in Appendix D. In the case of plant material inoculated with *A. tumefaciens* strains carrying the pBI121/Ac binary vector, the concentrations of kanamycin and glufosinate ammonium used in the tissue culture medium were lower than the minimum inhibitory concentrations determined previously (data not shown). This is because these two selection agents in combination inhibited the regeneration and growth of tobacco shoots more severely than when only one selection agent was used. Normally 100 mg/l kanamycin and 5 mg/l glufosinate ammonium were used when only one selection agent was added to the tissue culture medium but when both were used, these concentrations were reduced to 20 mg/l kanamycin and 1 mg/l glufosinate ammonium. When plant material was inoculated with *Agrobacterium* strains containing the pBI121/Ac binary vector, plant material was assayed for GUS activity (Jefferson, 1987) every seven days.

Shoots produced on tobacco leaf discs on medium containing selection agents were transferred to ½MS 30 medium (Appendix A) for rooting. The medium also contained the relevant selection agents. The transformed rooted plantlets produced, as well as untransformed control plants grown on medium lacking selection agents, were used to optimize assay systems for the *pat* gene. Some plantlets were hardened off in a mist bed and then sprayed with the herbicide Basta (1 g active ingredient/plant) to study the effect of the herbicide on the plants.

4.2.13 Analysis of transgenic tobacco plant material containing the *pat* gene

Transgenic Basta resistant tobacco plants and untransformed tobacco control plants were used to establish methods for analysing plant material transformed with the *pat* gene.

4.2.13.1 DNA extraction

DNA was extracted from plant tissues using a method based on that of Doyle and Doyle (1991) (Appendix D). Prior to DNA extraction, tissues were tested for the presence of *Agrobacterium* contamination which could lead to false positive results. The plant material was tested for contamination by streaking it gently over the surface of LA plates. The plates were incubated for 48 hours at 28°C. In cases where bacterial growth occured on plates, the plant material streaked on those plates was not used to set up assay systems. DNA was extracted from leaves of untransformed and transformed tobacco plants. The DNA concentration was determined by electrophoresis against DNA of known concentrations using a 2% Seakem agarose gel. DNA was also extracted from leaves of the soya bean cultivars Forrest and Hutton and the concentration of genomic DNA determined to ensure that DNA could be extracted from soya bean plant material using this method.

4.2.13.2 Polymerase chain reaction

The polymerase chain reaction (PCR) is a very powerful technique which allows *in vitro* amplification of specific DNA sequences from almost undetectable quantities of target DNA (Hamill *et al.*, 1991).

The polymerase chain reaction was carried out using primer sequences for the *pat* gene obtained from Dr Peter Eckes of AgrEvo AG. The primer sequences, excluding the *pat* gene promoter and terminator were:

Left primer (*pat* 5'):
5' ATG TCT CCG GAG AGG AGA CCA GTT GAG 3'

Right primer (*pat* 3'):
5' CTC AGA TCT GGG TAA CTG GCC TAA CTG 3'

DNA was extracted from tobacco plants transformed with the *A. tumefaciens* strain C58C1(pEHA101)(pHoe106/Ac) using the method described in section 4.2.13.1. Samples for PCR amplification were prepared by adding 50 ng template DNA to 5 µl $MgCl_2$ (25 mM stock), 5 µl 10 x buffer , 5 µl dNTP's (2 mM stock), 1 µl Taq polymerase (all obtained from Boehringer Mannheim), 1 µl *pat* 3' primer and 1 µl *pat* 5' primer. The reaction mix was made up to 50 µl with sterile distilled water and a layer of paraffin (50 µl) was added prior to PCR amplification.

DNA Samples were amplified in a Hybaid Omnigene thermal cycler. A denaturation period of five minutes at 94°C was followed by 30 cycles of amplification (annealing for two minutes at 60°C, extension for two minutes at 72°C, denaturation for one minute at 94°C). Final annealing and extension were achieved by incubating the samples for a further three minutes at 60°C and for five minutes at 72°C. Following PCR amplification, DNA samples were run on a 2% Seakem agarose gel at 75 volts for one hour to determine the presence of the *pat* gene amplification product. The size of the *pat* amplification product is 562 base pairs (bp).

4.2.13.3 DIG-labelling and detection

The DIG system uses digoxigenin to label DNA for hybridization and subsequent detection. The DIG-labelled probes are hybridized to membrane-bound DNA. These probes are then immuno-detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and then visualized using colourimetric or chemiluminescent substrates ("The Dig System User's Guide for Filter Hybridization", Boehringer Mannheim).

The 1.329 kb *pat* gene with promoter and terminator was excised from the plasmid pB2/35SAcK as described in Chapter 3. The *pat* gene was labelled with digoxigenin using the DIG DNA Labelling and Detection Kit obtained from Boehringer Mannheim. The methods described in Boehringer Mannheim's "The DIG System User's Guide for Filter Hybridization" were followed.

Initially, the colourimetric detection method using nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (X-phosphate) was used to detect DIG-labelled DNA. This method was later replaced because it was not sensitive enough to detect single gene copies in plant DNA. Instead, a chemiluminescent detection method was employed. The use of Lumigen PPD is described in Boehringer Mannheim's manual but Boehringer Mannheim's new product CDP-*Star*, which is highly sensitive and can detect single gene copies in plant DNA, was preferred.

DNA isolated from transformed and untransformed tobacco plants was blotted onto positively charged nylon membranes (Boehringer Mannheim) at concentrations of 1 µg and 500 ng. Bacterial DNA was blotted onto membranes at concentrations of 100 ng and 10 ng. The DNA on the membranes was UV cross-linked for three minutes before being processed according to the package insert obtained with CDP-*Star*.

4.3 RESULTS

4.3.1 Determination of concentrations of cefotaxime that controlled the growth of *A. tumefaciens* without affecting the growth of different soya bean explants

Experimental results obtained showed that 250 mg/l Cx included in the tissue culture medium did not control *A. tumefaciens* but 500 mg/l Cx did. A concentration of 1000 mg/l Cx inhibited development of callus on soya bean hypocotyls and regeneration from soya bean embryonic axes but did not have adverse effects on regeneration from cotyledons of soya bean. Routinely, a concentration of 500 mg/l Cx was used in all tissue culture media but in the case of soya bean cotyledons, a concentration of 1000 mg/l Cx was occasionally used when *Agrobacterium* contamination was severe.

4.3.2 Determination of concentrations of kanamycin that inhibited the growth of different untransformed soya bean explants

Experimental results obtained showed that 100 mg/l Km included in the tissue culture medium was sufficient to inhibit the development of callus on soya bean hypocotyls and the production of shoots from embryonic axes of soya bean, while 300 mg/l Km was required to inhibit regeneration from cotyledons of soya bean.

4.3.3 Determination of concentrations of glufosinate ammonium that inhibited the growth of different untransformed soya bean explants

It was found that the minimum inhibitory concentration (MIC) of glufosinate ammonium for soya bean shoots still attached to cotyledons was 80 mg/l. At this level shoots turned brown and died. At lower levels, some shoots died while others remained green and healthy. Shoots removed from cotyledons and cultured on rooting medium were prevented from rooting when concentrations of 1 mg/l glufosinate ammonium were included in the medium but only turned brown and died at concentrations of 20 mg/l glufosinate ammonium. Hypocotyls cultured on callusing medium produced some callus at concentrations of 1 to 3 mg/l glufosinate ammonium but did not produce any callus at glufosinate ammonium concentrations of 5 mg/l and above.

4.3.4 Preliminary experiments to determine which *A. tumefaciens* strains transform soya bean hypocotyls

No tumours were produced on soya bean hypocotyls using the virulent *A. tumefaciens* strains Ach5 and C58 and no GUS activity was detected in hypocotyl tissues inoculated with avirulent *A. tumefaciens* strains containing binary vectors. These observations could be due to the fact that these strains do not infect hypocotyl tissues of the soya bean cultivars tested or that the wounding and inoculation procedures were ineffective in inducing the virulence genes. Co-cultivation conditions may not have been suitable

for transformation or the *uid*A gene may have been present in tissues but not expressed due to the tissue specificity of the 35S promoter (Assad-Garcia *et al.*, 1992). Another factor to bear in mind is that tumours on soya bean are very small (Pedersen *et al.*, 1983) and may have been present but were not detected.

4.3.5 Transformation of cell suspension cultures of soya bean

Cell suspension cultures were assessed nine days after inoculation with C58C1(pGV2260)(pJIT119), once *Agrobacterium* had been controlled using 500 mg/l Cx, and showed GUS activity This means that transformation occurred and that the *uid*A gene was being expressed. Transformed cells expressing the *uid*A gene turn blue in the presence of the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-glu) (Figure 4.1). However, microcalli produced from the same cell suspesion culture grown on solid regeneration medium containing selective agents were GUS negative after 21 days of culture on solid medium i.e. 30 days after inoculation. This factor indicated that the expression of the *uid*A gene was not stable in these cells, although these microcalli grew on selection medium which would appear to contradict this. An explanation for this apparent contradiction could be that the *uid*A gene was not being expressed in these cells due to foreign gene inactivation but that the *npt*II gene was being expressed. This experiment was discarded after six weeks but this was more than likely too soon. It has been found in sunflower that several months of culture and selection are required to separate untransformed callus from transformed callus (S. Hearn, Personal Communication). A more probable explanation for the lack of GUS activity but growth of callus on selection medium may be that the untransformed callus could not be separated from the transformed callus in the early stages of the experiment, and that untransformed callus was assayed for GUS activity.

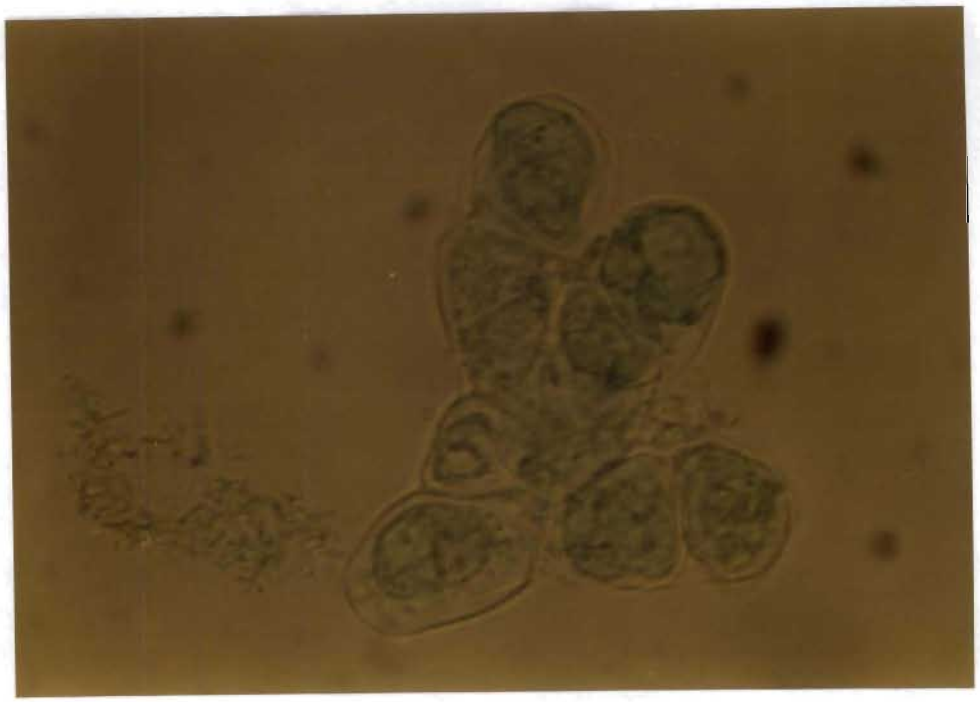

Figure 4.1 **GUS histochemical assay of soya bean (cv. Forrest) single cells transformed with the *uid*A gene (870 X Mag.). The blue colour indicates *uid*A gene expression**

4.3.6 Transformation of embryonic axes of soya bean

No evidence of transformation of soya bean embryonic axes was observed after using the wounding and inoculation methods described. No GUS activity was observed in embryonic axes at any stage. The production of shoots from the embryonic axes was completely inhibited on medium containing 100 mg/l kanamycin and no kanamycin resistant shoots were produced.

4.3.7 Transformation of hypocotyls of soya bean

No transformed soya bean hypocotyl callus was obtained in this study. This may have been due to problems with the selection of transformed cells. Callus produced on uninoculated hypocotyls is normally bright green when produced in the light. Hypocotyls inoculated with *Agrobacterium* and cultured in the light produced callus which was

yellow/brown in colour and showed signs of severe stress from the *Agrobacterium* infection. It was expected that on selection medium, transgenic callus would be bright green and non-transgenic callus would be brown. However, all hypocotyls inoculated with *Agrobacterium* produced yellow/brown callus so transformed and untransformed plant cells could not be distinguished from one another.

Selection using glufosinate ammonium is problematic in many plants, for example, tomato and chicory. This is because the phenolics produced by the dying untransformed cells often kill the transformed cells (G. Donn, Personal Communication). It is thought that this is also true for soya bean hypocotyl callus. A late selection is therefore necessary to allow transformed cells to multiply before selection so that these cells are able to withstand the effects of selection, namely the browning and death of adjacent cells. A late selection also allows the transformed callus to grow and become large enough to be cultured independently, away from the dying, untransformed material.

A seven day instead of a two day co-cultivation period, and a 1 in 100 000 instead of a 1 in 50 dilution of the *Agrobacterium* inoculum, resulted in much heathier green callus being produced after inoculation and subsequent culture on medium lacking selective agents. However, once this callus was transferred to medium containing selective agents, it turned brown indicating that no transformation had occurred.

The use of an *Agrobacterium* strain which carries a binary vector with the *npt*II gene, the *uid*A gene and the *pat* gene is of value for research purposes because early selection can be applied using kanamycin and the presence of transformed cells can be confirmed using the GUS assay (Jefferson, 1987). A late selection using glufosinate ammonium can then be applied to ensure that the *pat* gene is also being expressed. Soya bean hypocotyl callus however, was extremely sensitive to *Agrobacterium* inoculation. An early selection using kanamycin resulted in the death of the inoculated callus. It is thought that the callus required some time to recover from the stress of co-cultivation with *Agrobacterium* and a late selection using kanamycin or glufosinate ammonium is required to select for transformed soya bean callus.

It is clear from these experiments that in order to obtain transgenic soya bean hypocotyl callus, a very low *Agrobacterium* inoculum concentration such as a 1 in 100 000 dilution, together with a long co-cultivation period of approximately seven days must be used. A late selection is also required as early selection results in the death of all plant material whether it is transformed or not. A sample of callus assayed for GUS activity did not show any of the blue colouring which would indicate GUS activity but the sample size was very small. Only 1% of the total number of calli were assayed. If any transformed cells were present, they were probably eliminated by a too stringent selection regime. The transformation of hypocotyls therefore requires further detailed optimization if it is to be used as a system for testing *Agrobacterium* strains for their ability to transform soya bean.

4.3.8 Transformation of cotyledons of soya bean

4.3.8.1 Wounding of soya bean cotyledons for *A. tumefaciens* infection

The effect of wounding on the frequency of regeneration is shown in Table 4.1. The frequency of regeneration increased when cotyledons were wounded with sand or glass for 30 seconds but decreased in all other cases of wounding and desiccation as shown in Table 4.1. Although it would be useful to improve regeneration frequencies using wounding to stimulate regeneration, it is more important to wound tissues sufficiently to obtain transformation. For this reason, a more severe treatment for the wounding of soya bean cotyledons (60 seconds) was preferred. Later experiments however indicated that wounding may still not have been sufficient due to the lack of transformation. Cotyledons were thereafter wounded for two minutes using sand or powdered glass. The effect on regeneration was not quantified but regeneration still occurred.

Table 4.1 Effect of wounding on the frequency of shoot regeneration from cotyledons of soya bean (cv. Forrest)

Experiment	Treatment	Sample size (no. cotyledons)	% Regeneration[a]
1	Unwounded control	40	21
	Sand - 30 seconds	40	35
	Glass - 30 seconds	40	33
2	Unwounded control	40	42
	Sand - 60 seconds	40	25
	Glass - 60 seconds	40	37
3	Unwounded control	120	35
	Desiccation	120	23
	Cut in half	120	28

a: % of cotyledons that produced shoots *in vitro*

4.3.8.2 Transformation of soya bean cotyledons using *A. tumefaciens*

Wound sites on soya bean cotyledons were successfully transformed using the *Agrobacterium* strain C58C1(pGV2260)(pJIT119) but not when any of the other *Agrobacterium* strains were used. Transformation occurred when cotyledons were wounded by vortexing with sand for two minutes and co-cultivating cotyledons with C58C1(pGV2260)(pJIT119) for two days. Shoots regenerated from cotyledons of the cultivar Forrest were GUS positive but as all shoots were sacrificed for GUS assays, no kanamycin resistant plants were obtained. Although cotyledonary cells of the cultivars Forrest, Hutton and PNR577G showed GUS activity in numerous experiments with C58C1(pGV2260)(pJIT119), transformed shoots were only obtained in one experiment using the cultivar Forrest (Figures 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7).

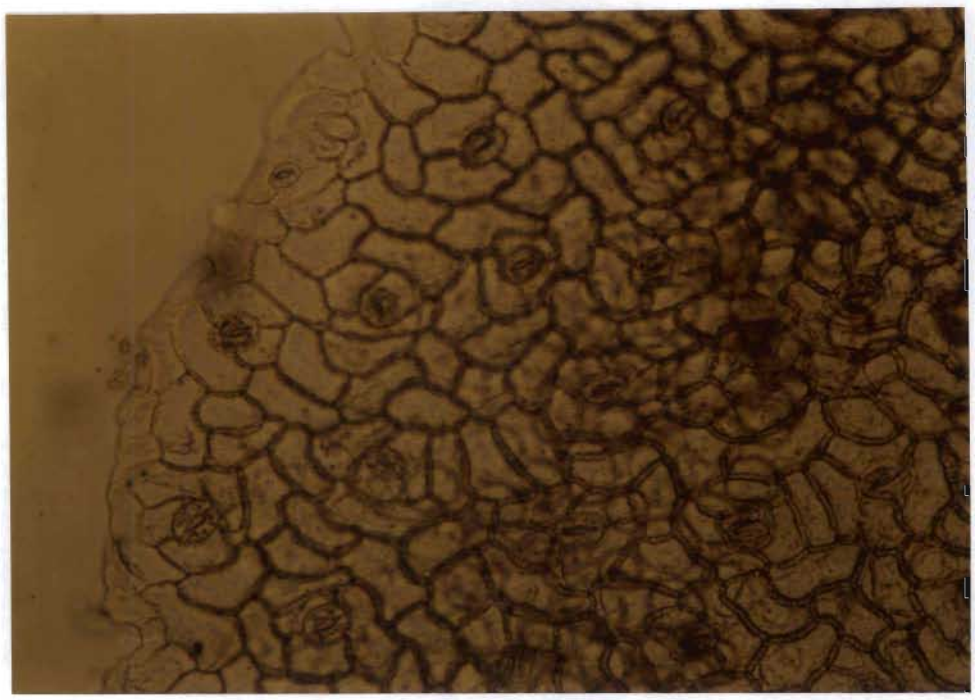

Figure 4.2 GUS histochemical assay of untransformed soya bean (cv. Forrest) cotyledon tissue (280 X Mag.). The lack of blue colour indicates the absence of *uid*A gene expression

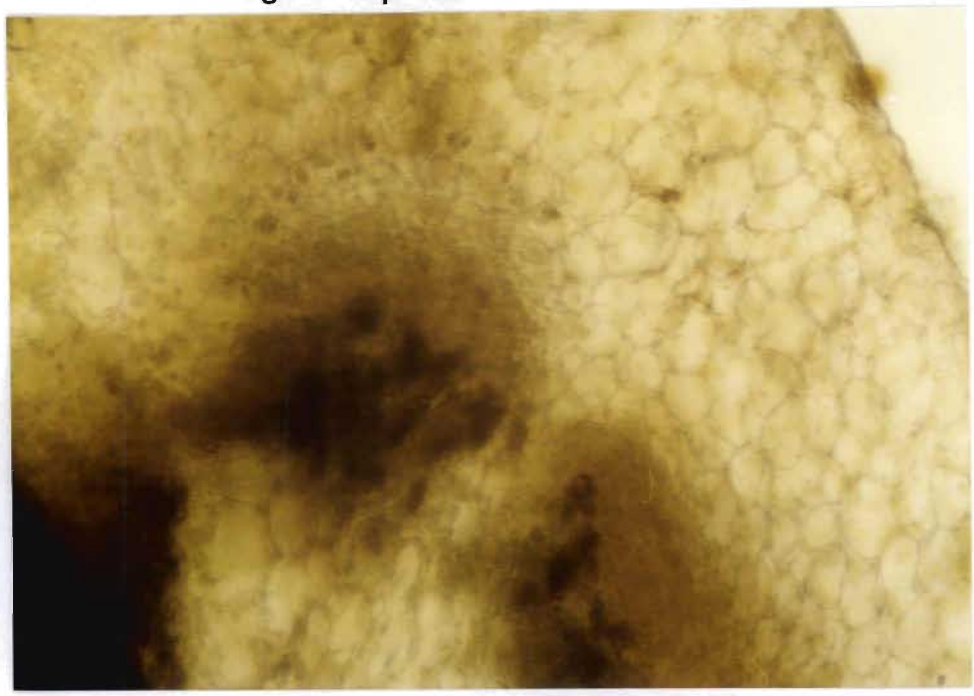

Figure 4.3 GUS histochemical assay of untransformed soya bean (cv. Forrest) leaf tissue (280 X Mag.). The lack of blue colour indicates the absence of *uid*A gene expression

Figure 4.4 A clump of *A. tumefaciens* cells attached to cotyledon cells of soya bean (cv. Forrest) (280 X Mag.). These bacterial cells contain and express the *uid*A gene and therefore give a blue colour reaction following the GUS histochemical assay

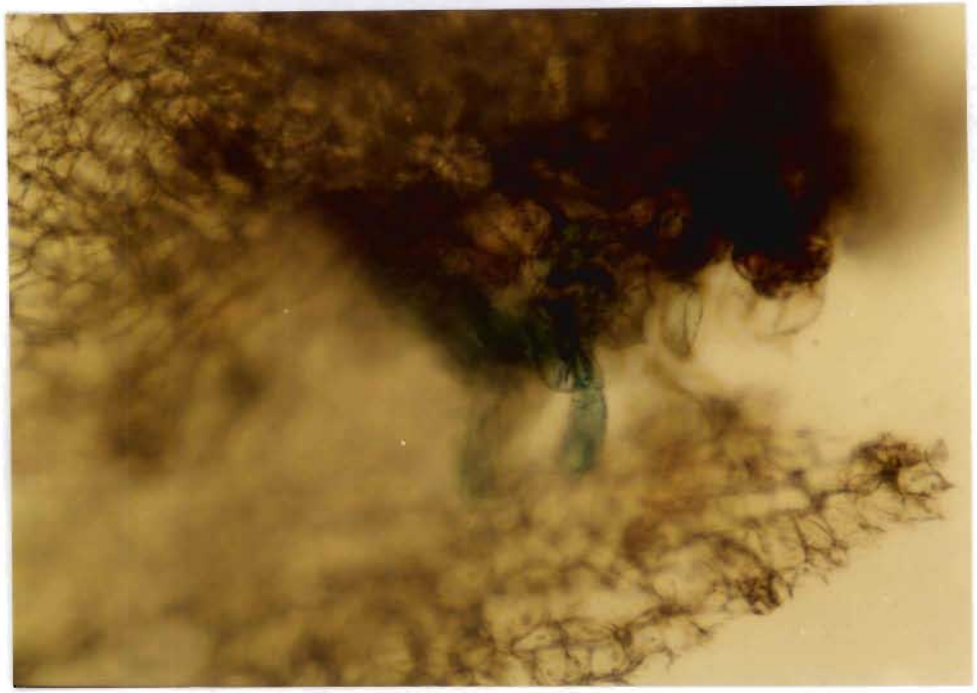

Figure 4.5 GUS histochemical assay of soya bean (cv. PNR577G) cotyledon tissue transformed with the *uid*A gene (280 X Mag.). The blue colour indicates *uid*A gene expression

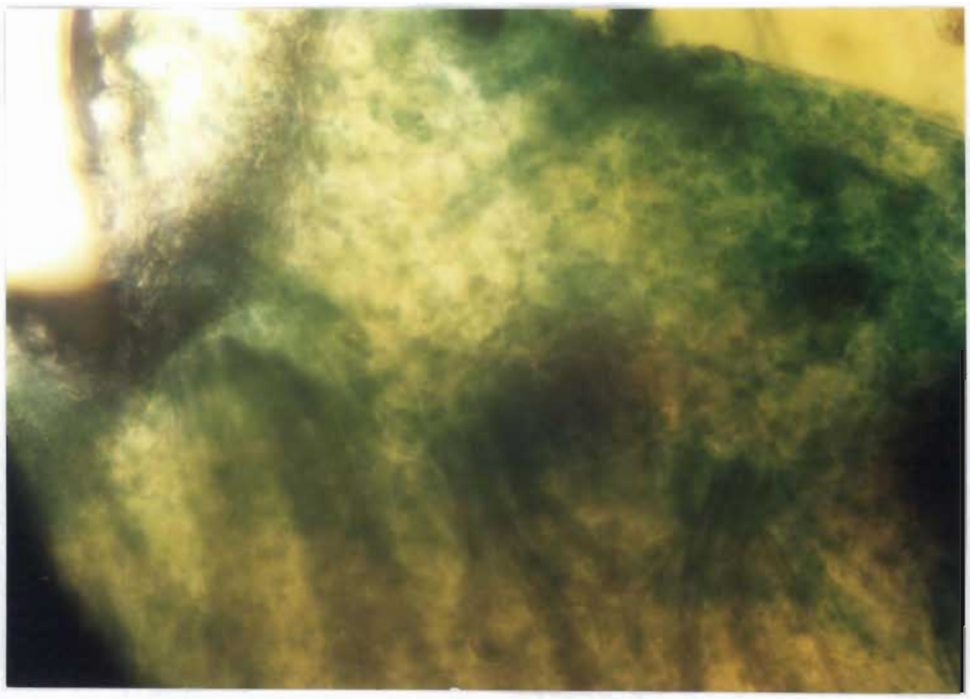

Figure 4.6 GUS histochemical assay of soya bean (cv. Forrest) leaf tissue transformed with the *uid*A gene (280 X Mag.). The blue colour indicates *uid*A gene expression

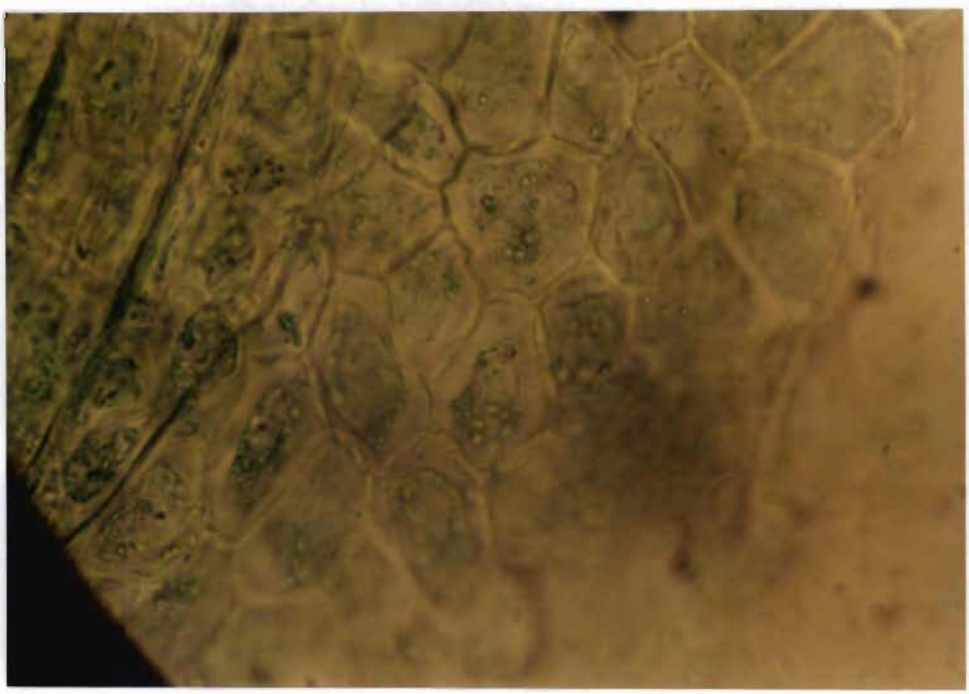

Figure 4.7 GUS histochemical assay of soya bean (cv. Forrest) leaf tissue transformed with the *uid*A gene (870 X Mag.). The blue colour indicates *uid*A gene expression

In transformation experiments using a modified feeder layer system, no transformation occurred with or without the use of the filter paper. It was hoped that the use of the modified feeder layer system would result in or enhance transformation but this did not occur. However, the lack of transformation indicated that there were problems with the transformation method used. Perhaps the strains that were tested were not able to transform soya bean tissues or the wounding procedures were inadequate. Regeneration occurred normally on medium lacking selective agents.

Shoots were regenerated from cotyledons of the cultivar Forrest when cotyledons were inoculated with the *Agrobacterium* strain C58C1(pGV2260)(pBI121/Ac) and cultured on regeneration medium lacking selective agents. This showed that *Agrobacterium* co-cultivation did not inhibit regeneration. However, when these shoots were placed on rooting medium containing selective agents, none survived indicating that no transformation had occurred. As with hypocotyl transformation, this may be due to problems with antibiotic selection. For example, if a chimaeric plant had been produced, the transformed cells may have been killed by the phenolics produced by the untransformed dying cells. A small sample of shoots (1%) assayed for GUS activity were also GUS negative however. This indicates that transformation did not occur and that this lack of transgenic tissues was not due to problems with selection but rather due to deficiencies in the wounding method or problems with strain specificity.

Although transgenic soya bean shoots regenerated from cotyledons were obtained, this was not by any means routine and further work is required to develop a successful genetic engineering system for soya bean.

4.3.9 Transformation of tobacco, a model system for plant genetic engineering, with *A. tumefaciens* strains containing the *pat* gene or the *pat* gene and the *uid*A gene

Transformed tobacco plants containing the *pat* gene were obtained using the *A. tumefaciens* strains C58C1(pEHA101)(pHoe106/Ac), C58C1(pGV2260)(pBI121/Ac),

LBA4404(pBI121/Ac) and EHA105(pBI121/Ac) (Figures 4.8, 4.9 and 4.10). Tobacco leaf discs co-cultivated with the strains C58C1(pMP90)(pHoe6/Ac) and C58C1(pATHV)(pHoe200/Ac) had to be discarded because the growth of the *Agrobacterium* could not be controlled. Subculturing to fresh medium containing cefotaxime was carried out once every two to three weeks instead of once a week and this was insufficient. Also, the concentration of cefotaxime in the fresh medium was 250 mg/l and perhaps 500 to 1000 mg/l would have resulted in better control of the *Agrobacterium*. Leaf discs inoculated with the strain C58C1(pMP90)(pBI121/Ac) did not produce any shoots on selection medium. It is possible that the experiment size was too small with only 15 leaf discs co-cultivated with this strain versus approximately 50 leaf discs in the experiments with the other *Agrobacterium* strains. Also, the selection was probably too severe. Initially, concentrations of 5 mg/l glufosinate ammonium and 100 mg/l kanamycin were used together to select for transgenic tobacco plants. It was then found that, in combination, only 1 mg/l glufosinate ammonium and 20 mg/l kanamycin was sufficient to inhibit regeneration of untransformed tobacco shoots.

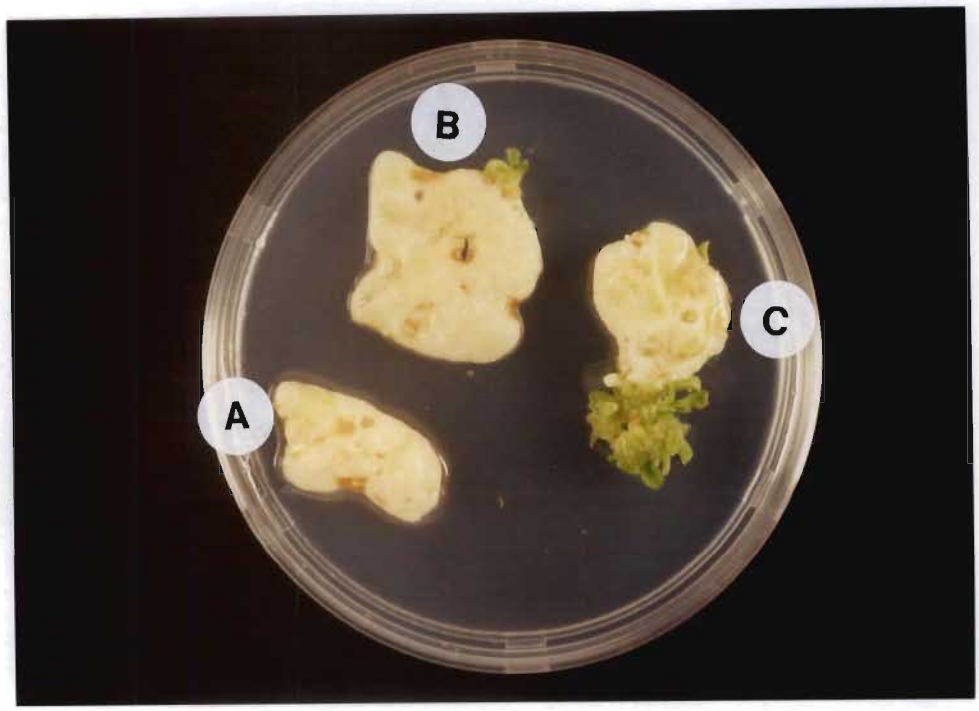

Figure 4.8 Untransformed and transformed tobacco (cv. A4) leaf discs cultured on regeneration medium containing 1 mg/l glufosinate ammonium and 20 mg/l kanamycin. A) Untransformed control leaf disc of tobacco, sensitive to glufosinate ammonium and kanamycin. B & C) Transformed shoots of tobacco resistant to glufosinate ammonium and kanamycin. The shoots were produced from leaf discs inoculated with the *A. tumefaciens* strain EHA105(pBI121/Ac)

Figure 4.9 Untransformed and transformed tobacco (cv. A4) plantlets cultured on rooting medium containing 5 mg/l glufosinate ammonium. A) Transformed tobacco plant resistant to glufosinate ammonium. This plant was produced by inoculating leaf discs with the *A. tumefaciens* strain C58C1(pEHA101)(pHoe106/Ac) and then transferring the resulting shoots to rooting medium. B) Untransformed control tobacco plant sensitive to glufosinate ammonium

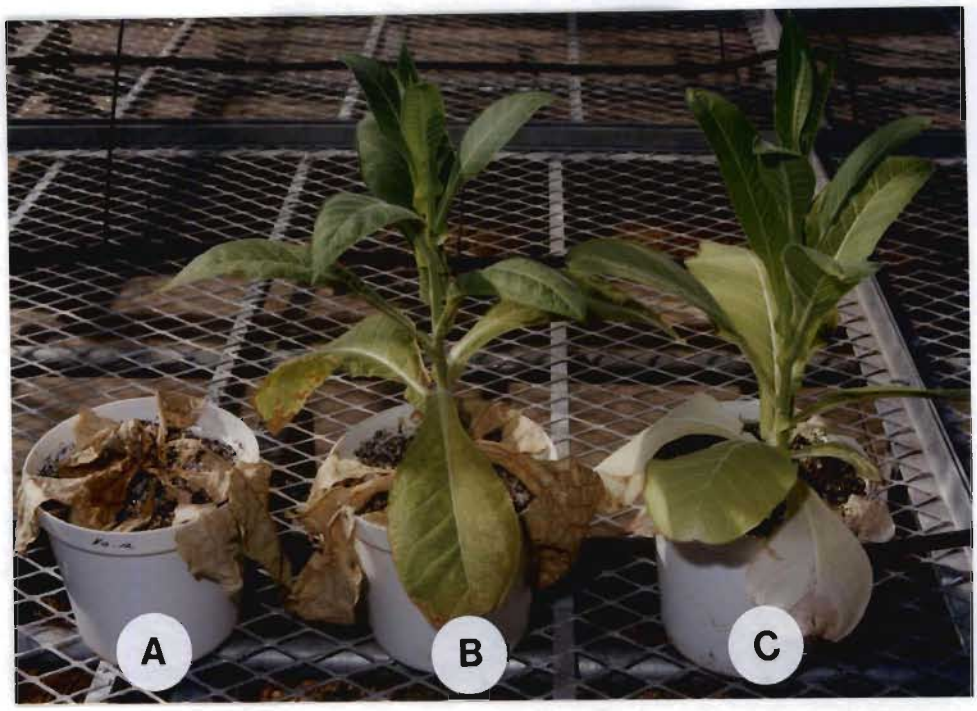

Figure 4.10 Untransformed and transformed hardened off tobacco (cv. A4) plants five days after spraying with the herbicide Basta (1 g active ingredient/plant). A) Untransformed control tobacco plant sensitive to Basta. B) Transformed tobacco plant resistant to Basta. C) Untransformed control plant not sprayed with Basta

4.3.10 Analysis of transgenic tobacco plant material containing the *pat* gene

4.3.10.1 DNA extraction

The concentration of plant DNA extracted from tobacco leaves was approximately the same as DNA extracted from leaves of the soya bean cultivar Forrest (20 µg DNA/g leaf material). However, in the case of the soya bean cultivar Hutton, only 5 µg DNA/g leaf material was extracted. The DNA extracted could be used successfully for the polymerase chain reaction. It must be noted, however, that DNA extracted from plants using this method cannot be used for Southern blots due to the large quantities of DNAases still present in the preparation (M. O'Kennedy, Personal Communication).

4.3.10.2 Polymerase chain reaction

Figure 4.11 shows the results of PCR analysis to determine the presence of the *pat* gene in tobacco. Tobacco DNA transformed with the *Agrobacterium* strain C58C1(pEHA101)(pHoe106/Ac) was used for PCR analysis. The *pat* gene amplification product ran at the expected size of 562 bp.

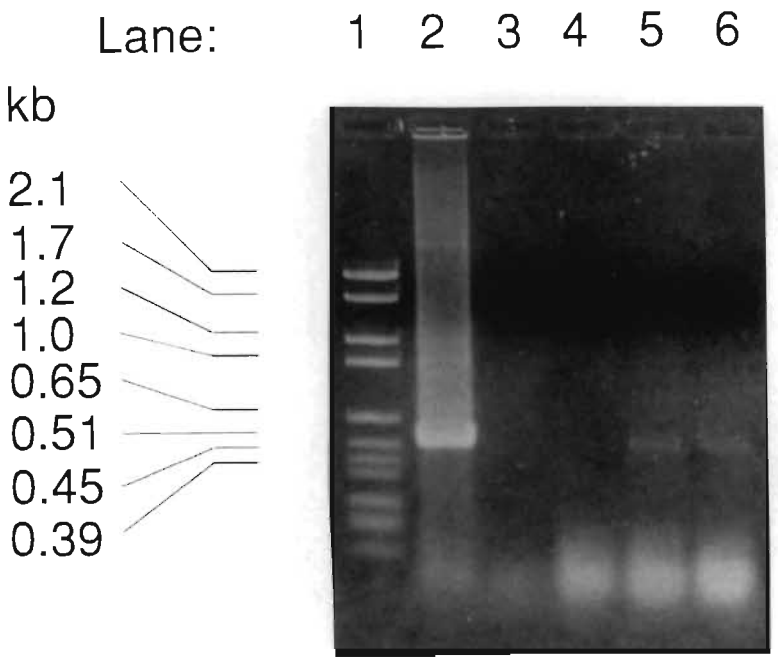

Figure 4.11 Agarose gel showing the products of PCR amplification of the *pat* gene. Lane 1: λ VI marker (Boehringer Mannheim); Lane 2: Control DNA - pB2/35SAcK DNA containing the *pat* gene; Lane 3: Control - no template DNA; Lane 4: Control - untransformed tobacco DNA; Lane 5: Transformed tobacco DNA containing the *pat* gene - plant 1; Lane 6: Transformed tobacco DNA containing the *pat* gene - plant 2. The *pat* gene product is visible, as expected, in lanes 2, 5 and 6

4.3.10.3 DIG-labelling and detection

It was found that colourimetric detection with NBT and X-phosphate was not sensitive enough to detect single *pat* gene copies in plant DNA. The gene could not be detected in plant DNA even at DNA concentrations of 1.5 µg per dot blot. The large amount of DNA required makes this method impractical. Ideally, it is not desirable to sacrifice an entire plant to confirm that it is transformed. A concentration of control plasmid

pB2/35SAcK DNA of 20 ng could be detected using the colourimetric detection method indicating that this DNA probing method does work but that it is not sensitive enough for our purposes. The chemiluminescent detection method is more sensitive and 1 pg of plasmid DNA has been detected in 500 ng of plant DNA per dot blot in this study. This is the approximate size of a single gene copy ("The DIG System User's Guide for Filter Hybridization", Boehringer Mannheim).

The *pat* gene was detected in DNA extracted from transformed tobacco plants inoculated with C58C1(pEHA101)(pHoe106/Ac) (Figure 4.12). However, a problem with the chemiluminescent detection method was that non-specific binding to the untransformed tobacco control DNA occurred. This could be due to the probe detecting the promoter and terminator of the *pat* gene but it is more likely that the washing steps did not remove unbound probe from the DNA effectively. The non-specific binding of the probe could also be due to the fact that the plant DNA was extracted using the method described in section 4.2.13.1. It is known that DNA extracted from plants in this manner is not of sufficient quality for use in DIG-labelling and detection of Southern blots. A DNA extraction method which incorporates a phenol-chloroform step is required for the chemiluminescent detection method of Southern blots (M. O'Kennedy, Personal Communication). It is probable that this is also true for chemiluminescent detection of dot blots. Therefore, before this assay system can be used routinely to detect the *pat* gene in transformed plant DNA, the system must be optimized. The *pat* gene may have to be cut out of pB2/35SAcK again without the promoter and terminator, the washing steps may have to be modified to remove unbound probe, or another plant DNA extraction method will have to be used.

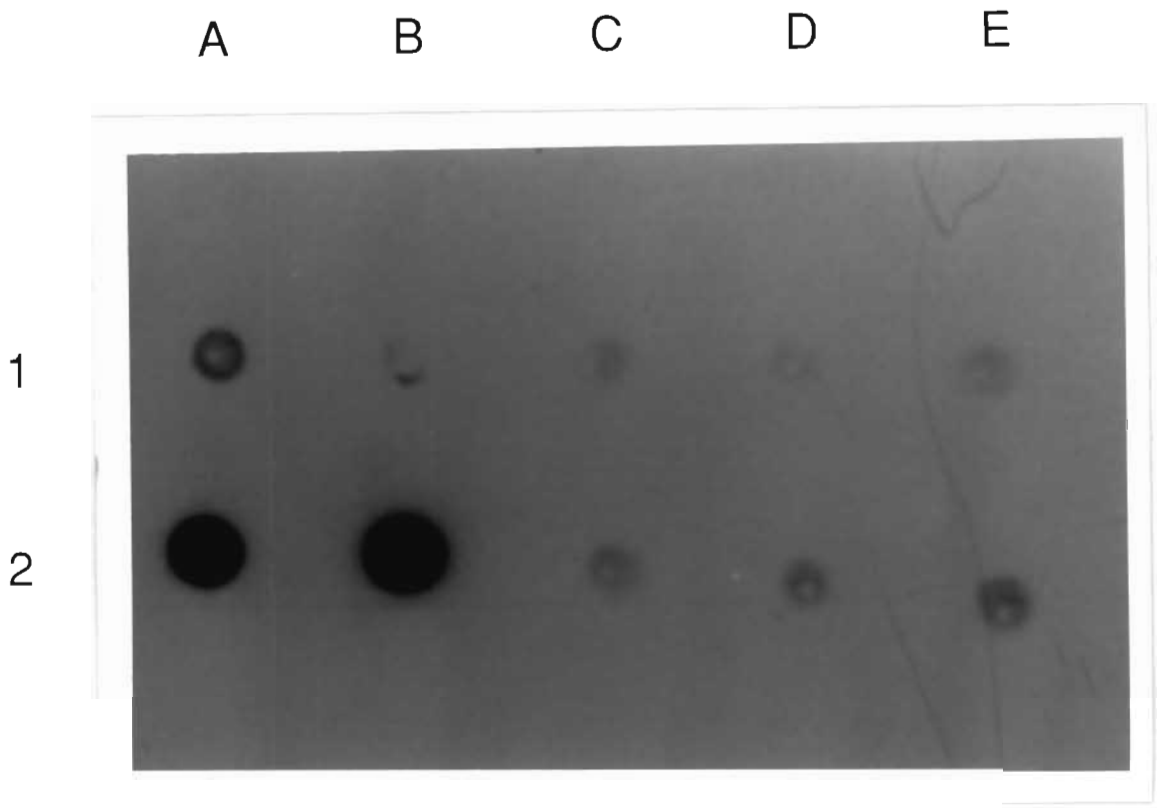

Figure 4.12 Chemiluminescent dot blot detection of the *pat* gene in bacterial DNA and transgenic plant DNA using a digoxigenin-labelled DNA probe. A1: 10 pg control labelled bacterial DNA obtained from Boehringer Mannheim; A2: 100 pg control labelled DNA obtained from Boehringer Mannheim; B1: 10 pg pB2/35SAcK DNA; B2: 100 pg pB2/35SAcK DNA; C1: 500 ng transformed tobacco DNA from plant 1; C2: 1 µg transformed tobacco DNA from plant 1; D1: 500 ng transformed tobacco DNA from plant 2; D2: 1 µg transformed tobacco DNA from plant 2; E1: 500 ng untransformed tobacco DNA detected despite the *pat* gene being absent; E2: 1 µg untransformed tobacco DNA detected despite the *pat* gene being absent

4.4 DISCUSSION

In order to control the growth of *Agrobacterium* and to select transformed plant cells from untransformed cells, it was necessary to determine the minimum inhibitory concentrations of cefotaxime, kanamycin and glufosinate ammonium which inhibited the growth of untransformed soya bean explants. Of particular importance was the observation that notably different concentrations of glufosinate ammonium inhibited the growth of various soya bean explants. Different concentrations inhibited the growth of soya bean shoots on cotyledons, the rooting of soya bean shoots and the production of callus on hypocotyls. It is obvious that different types of explants have different sensitivities to glufosinate ammonium. In the case of shoots still attached to the cotyledons for example, the cotyledons protect the shoots to some extent from the toxic effects of glufosinate ammonium. Also of interest and not unexpected was the fact that if kanamycin and glufosinate ammonium were used in combination in selection medium, concentrations of both compounds could be lower than the usual MIC's and still inhibit the growth of the plant material due to the greater toxic effect on the material.

Cell suspension cultures could easily be transformed with disarmed *Agrobacterium* strains but foreign gene expression was not stable. This may be due to altered foreign gene expression which can be caused by a number of factors. Some of these factors include the position and the number of integrations of the foreign gene within the plant genome. DNA methylation and gene deletions may result in the inhibition of foreign gene expression. Qualitative changes can also occur resulting in altered organ-specific patterns of expression. The developmental stage of the plant, light intensities and supplements such as sucrose and plant hormones included in the tissue culture medium may also affect foreign gene expression (Herrera-Estrella and Simpson, 1988; Weber *et al.*, 1990; Assad-Garcia *et al.*, 1992). The transformation of soya bean cell suspension cultures was also of limited value because plants could not be regenerated from these cultures. Cell suspensions could be useful for the testing of disarmed *A. tumefaciens* strains containing binary vectors to determine whether these strains transform soya bean but the method for obtaining stably transformed callus, perhaps by selection over several months, requires further careful optimization.

Although shoots could be produced from embryonic axes in this study, embryonic axes could not be transformed using any of the *Agrobacterium*-mediated transformation methods tested, possibly because only one, perhaps ineffective, *Agrobacterium* strain was used. It is possible that a particle gun-based transformation method would be useful in the transformation of embryonic axes. Actively dividing explants such as embryonic axes are usually more likely to be transformed using the particle gun than less actively dividing explants such as cotyledons (M. O'Kennedy, Personal Communication)

Although no tumours were found on soya bean hypocotyls infected with the wild-type *Agrobacterium* strains Ach5 and C58, this was not entirely unexpected. Tumours on soya bean hypocotyls were reported to be very small and use of a microscope is required to confirm the presence of galls (Pedersen *et al.*, 1983). A microscope was not used in this study. It does appear however, that these wild-type strains cannot transform soya bean hypocotyls. This implies that disarmed *Agrobacterium* strains will also not transform soya bean hypocotyls. This was confirmed in this study. Transformed callus from soya bean hypocotyls was not obtained using disarmed *Agrobacterium* strains. The problems of co-cultivation and selection were better understood after this work. The transformation of hypocotyls could be of use in establishing assay systems for foreign gene detection and expression, and also to test transconjugant *Agrobacterium* strains but no transgenic callus was produced from soya bean hypocotyls in this study.

Because a routine method was available for the regeneration of fertile plants from cotyledons, cotyledons were used extensively as the explant material in transformation experiments. Although transformation of wound sites on cotyledons (GUS positive areas) was obtained in several experiments, a routine method for the transformation of cotyledons was not developed. Only the *Agrobacterium* strain C58C1(pGV2260)(pJIT119) was able to transform cotyledonary cells of soya bean, so methods of reducing the strain effect, for example, by using longer co-cultivation times, could be useful (G. Donn, Personal Communication).

The novel binary vector constructed in this study to facilitate the development of an *Agrobacterium*-mediated transformation system for soya bean, was not successful in transforming soya bean plant material. This was largely due to the difficulties encountered with the genetic engineering of soya bean and not due to faults with the new construct. Although transformed shoots resistant to kanamycin were regenerated from mature soya bean cotyledons, it was not possible to obtain transgenic soya bean plant material resistant to the herbicide Basta. As a result, tobacco which is a model system for plant genetic engineering was used in this study. Tobacco leaf discs were successfully transformed to produce transgenic plants resistant to Basta. This transgenic material was used to optimize assays for the analysis of material containing the *pat* gene. A DNA extraction method for soya bean and tobacco was optimized. The presence of the *pat* gene in transgenic tobacco tissues was confirmed using PCR. A DIG-labelling and hybridization method to detect foreign genes in plant DNA was also examined. The chemiluminescent DIG detection method was found to be more sensitive than colourimetric DIG detection and could be used to analyse DNA from transgenic plant material. However, the DIG-labelled DNA probe hybridized non-specifically with DNA extracted from both transformed and untransformed tobacco plants and this procedure therefore requires further optimization.

Not tested in this study but available for the detection of the presence and expression of the *pat* gene, is the phosphinothricin acetyltransferase (PAT) assay. The details of the PAT assay are given in Appendix D. This assay is a simple radio-active assay which confirms the presence of the *pat* gene in plant material through the activity of its gene product, phosphinothricin acetyltransferase. Plants convert radio-active L-phosphinothricin (PPT) or radio-active acetyl co-enzyme A to radio-active N-acetyl phosphinothricin which can then be visualized on an autoradiograph (G. Donn, Personal Communication). The PAT assay is a useful method for the detection of the *pat* gene because only 50 mg of plant material are required for the assay. Entire plants therefore do not need to be sacrificed for analysis as is the case with Southern blots where enormous quantities of plant DNA are required.

In this study, *Agrobacterium*-mediated transformation of soya bean was the focus of attention despite the proven success of particle gun technology for the transformation of soya bean. This was due to patent restrictions and client considerations. From the research conducted in this study however, it is clear that *A. tumefaciens*-mediated transformation of soya bean is extremely difficult. This is especially evident when the problems encountered with soya bean transformation are compared with the ease with which transgenic tobacco plants could be produced. Although a number of soya bean cultivars, different soya bean explants, a variety of wounding techniques and numerous wild-type and disarmed *Agrobacterium* strains were tested in this study, transformation of soya bean occurred only rarely.

Soya bean has been found to be extremely recalcitrant to regeneration and transformation and very few research groups world-wide have been able to successfully genetically engineer soya bean on a routine basis. At present, only the research group from Agracetus can genetically engineer soya bean in a variety-independent fashion for commercial purposes. This method is patented (Christou, 1994). Finer and McMullen (1991) have also reported a routine method for the production of transgenic soya bean from embryonic cell suspension cultures but this method has not been commercialized as yet. Both these groups make use of particle gun technology. Only one group (Townsend and Thomas, 1994) has obtained routine transformation of soya bean using *A. tumefaciens* but this method is protected by patent rights.

A recommendation for future work in the area of soya bean transformation is that the particle gun is used to transform cotyledons of soya bean with plasmid DNA or that the particle gun is used to wound cotyledons prior to co-cultivation with *A. tumefaciens* according to the method of Bidney *et al.* (1992) which was successfully used to transform sunflower, another recalcitrant plant. From publications on the transformation of soya bean and from the research conducted in this thesis, all indications are that *Agrobacterium*-mediated transformation is not very successful for the production of transgenic soya bean plants and that particle gun methods are more efficient for the production of genetically engineered soya bean on a routine basis.

CHAPTER 5

CONCLUSIONS

The aim of this study was to develop a novel *Agrobacterium*-mediated transformation system for soya bean, specifically to transfer the *pat* gene for Basta resistance into this crop. An *Agrobacterium*-mediated transformation system requires an *in vitro* regeneration system, a wounding method that allows *Agrobacterium* attachment but does not abolish regeneration, suitable genes in a vector system for transfer into the plants, and a selection system to identify transgenic plant material. Although a routine transformation method for soya bean using *Agrobacterium* was not achieved in this study, the problems associated with developing such a system for a recalcitrant crop were comprehensively investigated and are now fully appreciated by the author. The legal steps which follow on from the successful production, testing and release of a transgenic crop are also considered in this chapter.

Soya bean is known to be the most difficult *Glycine* species to regenerate. Explants do not readily produce roots, making further cultivations of transgenic material almost impossible (Townsend and Thomas, 1994). For this reason, the establishment of a routine regeneration method which results in rooted plantlets which can be hardened off, and can flower and set seed, is of critical importance in a genetic engineering system for this crop.

Despite the recalcitrant nature of soya bean and the numerous problems encountered, a routine method for the *in vitro* regeneration of fertile plants from mature soya bean cotyledons was developed. A variety of soya bean explants were investigated for their potential to regenerate. Of the many explants tested only soya bean cotyledons proved to be of use in the regeneration of shoots in an efficient routine genotype-independent manner. Shoots could also be produced from embryonic axes of soya bean but this was less efficient than the regeneration of shoots from cotyledons.

Although a novel routine transformation method for soya bean was not developed in this study, some transgenic soya bean plant material was obtained (cell suspension cultures, cotyledons and shoots regenerated from cotyledons). Soya bean is extremely recalcitrant to *Agrobacterium*-mediated transformation. This is more than likely due to the fact that soya bean has a limited susceptibility to *Agrobacterium* which is soya bean genotype and *Agrobacterium* strain dependent. Only one group world-wide (Pioneer Hi-Bred International) has reported a genotype-independent *Agrobacterium*-mediated transformation method for soya bean (Townsend and Thomas, 1994). Because of this, and the fact that in this study only limited transformation of soya bean using *A. tumefaciens* has been achieved, it is suggested that the particle gun be used in future research for the development of a routine genetic engineering system for soya bean.

In the initial stages of this research project, only the use of the particle gun to transform soya bean had been patented. The existence of this patent right was the main reason for the use of an *Agrobacterium*-mediated approach to the transformation of soya bean. In 1994 however, Agracetus was awarded the rights to all genetically engineered soya beans in Europe regardless of the methods used to produce transgenic soya bean plants. A similar claim is pending in the United States. The Agracetus soya bean patent and another Agracetus patent on the genetic engineering of cotton are both being challenged because of their broadness (Mestel,1994). Although the soya bean patent does not cover South Africa as yet, it is probably only a matter of time before it does, unless the patent is rejected world-wide. Whichever transformation method is used to genetically engineer soya bean, licences and fees will have to be negotiated with Agracetus. The use of the particle gun method is therefore preferred as it is more likely to result in transgenic soya bean plants than are *Agrobacterium*-mediated transformation methods.

A binary vector plasmid containing the *uid*A gene and the *pat* gene was constructed in this study and is of value in the development of transformation systems for many plants. It is useful for research purposes to use the GUS marker for early detection of transformation and *npt*II or *pat* genes for later selection. For commercial purposes however, plants containing as few foreign genes as possible are desired. The new

construct was conjugated into four different disarmed *A. tumefaciens* strains. Three of these strains were successfully used to produce transgenic tobacco plants resistant to kanamycin, Basta and which expressed the *uid*A gene.

Assay systems for the analysis of transgenic material containing the *pat* gene were established using tobacco transformed with the *Agrobacterium* strain C58C1(pEHA101)(pHoe106/Ac). The assay systems can be applied to any transgenic plant material containing the *pat* gene.

The development of gene transfer techniques for soya bean is of commercial interest because these techniques facilitate the development of cultivars with new traits. Novel traits that could be introduced into soya bean through genetic engineering to improve the crop include herbicide, disease and insect resistance, and drought tolerance. As it is a nitrogen-fixing legume, it may also be possible to manipulate the symbiotic plant-*Rhizobium* relationship and this could have an impact on yields and production costs. Probably the most important area of soya bean improvement lies in the manipulation of traits such as quantity and quality of seed protein and oil. Improvements in these traits would increase the nutritional value of the crop. In the future, soya bean genotypes with greater protein and/or oil content and genotypes with significantly altered oil composition such as a lower saturated fat content are expected (Hinchee *et al.*, 1988; Widholm, 1988; Christou *et al.*, 1990; Townsend and Thomas, 1994).

Once transgenic plants have been obtained, a further problem is the regulation of their release and testing. One aspect of this is patent protection. Patent protection is one of the ways in which a company investing in agricultural biotechnology is ensured of an acceptable return on investment. Patenting conditions vary from country to country. For example, plant and animal varieties can be patented in the United States and Japan but not in Europe (or South Africa). However, the European based Union for the Protection of New Plant Varieties (UPOV) of which South Africa is a member, has recently strengthened its constitution to include protection for "essentially derived varieties". This means that varieties with single gene modifications due to genetic engineering may be protected under plant breeders rights. In South Africa this means,

among other things, that new varieties are protected for 20 years (25 years for fruit trees) and breeders can sue for damages in cases of infringement (Webster and Dyer, 1994).

Legislation, like patent protection varies considerably from country to country. Countries like Germany possess extremely restrictive biotechnology legislation, although consideration is being given to relaxation of German laws at present. Countries such as Pakistan, Kenya, Zimbabwe and South Africa have no legislation at all for the control and release of genetically modified organisms. However, countries like India and South Africa have established safety committees to monitor the release of transgenic organisms (Webster and Dyer, 1994).

In South Africa, a safety committee called the South African Committee for Genetic Engineering (SAGENE) was established in the late 1970's and has played an essential role in the control and release of genetically modified organisms. However, this committee has neither the mandate nor the resources to enforce the guidelines established by the committee. SAGENE acts as the national advisory body on recombinant DNA technology and serves the interests of the scientific community and the public in this regard. The committee advises any person concerned with recombinant DNA research, on guidelines for the application of recombinant technology, as well as the possible effect on the environment following the release of organisms with recombinant DNA. SAGENE also advises any Minister, Statutory or State body, or industry on any form of legislation or controls pertaining to the importation and/or release into the environment of organisms with recombinant DNA. Various guides dealing with recombinant DNA technology can be obtained from SAGENE, Foundation for Research and Development, PO Box 2600, Pretoria, 0001. These include "Guidelines and notification procedure for laboratory containment of genetically manipulated organisms", "Guidelines for the categorisation of genetic manipulation experiments", and "Guidelines and notification procedure for the large-scale use of genetically manipulated organisms" (Webster and Dyer, 1994).

SAGENE has no power or authority to approve or prohibit the importation, trial release or general release of a genetically modified organism and can only advise the appropriate regulatory body. An example of the type of questionnaire given to applicants wanting to import or release genetically modified organisms (Appendix E). however, indicates that SAGENE is attempting to minimize any risks associated with the release of these organisms (Webster and Dyer, 1994). From this questionnaire it is also clear that although the development of a genetic engineering system for a particular plant and the development of constructs containing suitable foreign genes for transfer into plants are long-term projects, other important phases follow. Once transgenic plants have been obtained *in vitro*, greenhouse trials and progeny analyses are required to determine the gene segregation ratios. Field trials must then be carried out for further analyses and only then can these modified plants be introduced into plant breeding programmes.

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APPENDIX A

PLANT TISSUE CULTURE MEDIA

MS Medium (Murashige and Skoog, 1962)

	g/l	
Major inorganic nutrients		
NH_4NO_3	33	
KNO_3	38	Add 50 ml stock per litre
$CaCl_2.2H_2O$	8.8	of medium
$MgSO_4.7H_2O$	7.4	
KH_2PO_4	3.4	
Trace Elements		
KI	0.166	
H_3BO_3	1.24	
$MnSO_4.4H_2O$	4.46	Add 5 ml stock per
$ZnSO_4.7H_2O$	1.72	litre of medium
$Na_2MoO_4.2H_2O$	0.05	
$CuSO_4.5H_2O$	0.005	
$CoCl_2.6H_2O$	0.005	
Iron Source		
$FeSO_4.7H_2O$	5.56	Add 5 ml stock per litre
$Na_2EDTA.2H_2O$	7.46	of medium
Organic supplement		
Myo-Inositol	20	
Nicotinic acid	0.1	Add 5 ml stock per litre
Pyridoxine-HCl	0.1	of medium
Thiamine-HCl	0.1	
Glycine	0.4	
Carbon source		
Sucrose	0 to 30	

B5 Medium (Gamborg *et al.*, 1968)

	g/l	
Major inorganic nutrients		
$(NH_4)_2SO_4$	2.6	
KNO_3	60	Add 50 ml stock per litre
$CaCl_2.2H_2O$	3	of medium
$MgSO_4.7H_2O$	10	
$NaH_2PO_4.H_2O$	3	
Trace elements		
$MnSO_4.H_2O$	2	
$ZnSO_4.7H_2O$	0.4	
H_3BO_3	0.6	Add 5 ml stock per litre
KI	0.15	of medium
$Na_2MoO_4.2H_2O$	0.05	
$CoCl_2.6H_2O$	0.005	
$CuSO_4.5H_2O$	0.005	
Iron source		
$FeSO_4.7H_2O$	5.56	Add 5 ml stock per litre
$Na_2EDTA.2H_2o$	7.46	of medium
Organic supplement		
Myo-Inositol	20	
Nicotinic acid	0.2	Add 5 ml stock per litre
Pyridoxine-HCl	0.2	of medium
Thiamine-HCl	2	
Carbon source		
Sucrose	0 to 30	

½MS (Murashige and Skoog, 1962)

Half the inorganic salt concentration of MS medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock

½ B5 (Gamborg *et al.*, 1968)

Half the inorganic salt concentration of B5 medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l B5 vitamin stock

½ MS + x mg/l BA (Wright *et al.*, 1986)

Half of the inorganic salt concentration of MS medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
x mg/l BA where x = 0.5 or 1

MS + x mg/l BA (Wright *et al.*, 1986)

MS basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
x mg/l BA where x = 0.5 or 1

MS + x mg/l BA + y mg/l NAA (experimental)

MS basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
1 g/l thiamine
x mg/l BA where x = 0, 1, 2 or 3
y mg/l NAA where y = 0.5, 2 or 4

B5 + x mg/l BA + y mg/l 2,4-D (experimental)

B5 basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l B5 vitamin stock
0.5 g/l casein hydrolysate
1.67 mg/l thiamine
3.66 mg/l nicotinic acid
x mg/l BA where x = 0.5
y mg/l 2,4-D where y = 1 or 1.5

MSC (Draper *et al.*, 1988)

MS basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
2 mg/l NAA
1 mg/l BA

MSC liquid medium (Draper *et al.*, 1988)

MS basal medium
30 g/l sucrose
5 ml/l MS vitamin stock added before autoclaving
2 mg/l NAA added before autoclaving
1 mg/l BA added before autoclaving

MSS (Draper *et al.*, 1988)

MS basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
1 mg/l BA
0.1 mg/l IAA

B5S (experimental)

B5 basal medium
0 to 30 g/l medium
6 g/l agar
5 ml/l B5 vitamin stock
1 mg/l BA
0.1 mg/l IAA

EB (Barwale *et al.*, 1986)

MS basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
1.67 mg/l thiamine
3.66 mg/l nicotinic acid
x mg/l NAA where x = 4, 8 or 12

EB B5 (experimental)

B5 basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l B5 vitamin stock
1.67 mg/l thiamine
3.66 mg/l nicotinic acid
x mg/l NAA where x = 8 or 24

OR (Barwale *et al.*, 1986)

MS major salts
4 x concentration of MS minor salts
MS iron source
0 to 30 g/l sucrose
6 g/l agar
5 ml/l B5 vitamin stock
1.67 mg/l thiamine
0.04 mg/l NAA
x mg/l BA where x = 1, 2, 3 or 5

MSR (Barwale *et al.*, 1986)

MS basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
0.4 mg/l BA
0.04 mg/l IBA

R5 (Barwale *et al.*, 1986)

MS basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l MS vitamin stock
0.002 mg/l BA
2 mg/l IBA
1.7 mg/l GA_3

B5 BA (Hinchee *et al.*, 1988)

B5 basal medium
0 to 30 g/l sucrose
6 g/l agar
5 ml/l B5 vitamin stock
x mg/l BA where x = 0.5, 1, 2 or 3

APPENDIX B

BACTERIOLOGICAL MEDIA, BUFFERS AND SOLUTIONS

Luria agar (Sambrook *et al.*, 1989)

10 g/l tryptone
5 g/l yeast extract
10 g/l NaCl
15 g/l agar
pH 7.2

Luria broth (Sambrook *et al.*, 1989)

10 g/l tryptone
5 g/l yeast extract
10 g/l NaCl
pH 7.2

Lysis solution (Armitage *et al.*, 1988)

1.25 ml 2 M tris-HCl (pH 8)
2 ml 0.5 M EDTA
9.01 g glucose
0.10 g lysozyme

Made up to 100 ml with distilled water

Tris-EDTA buffer (TE) (Armitage *et al.*, 1988)

2 ml 0.5 M EDTA (pH 8)
10 ml 1 M tris (pH 8)

Made up to 1 litre with distilled water

Cesium Chloride Stock (Armitage *et al.*,1988)

50 ml TE
64 g CsCl

Adjusted to refractive index 1,402

10 x Tris-acetate buffer (TAE) (Sambrook *et al.*, 1989)

48.4 g tris
11.42 ml glacial acetic acid
20 ml 0.5 M EDTA (pH 8)

Made up to 1 litre with distilled water

5 x Tris-borate buffer (TBE) (Sambrook *et al.*, 1989)

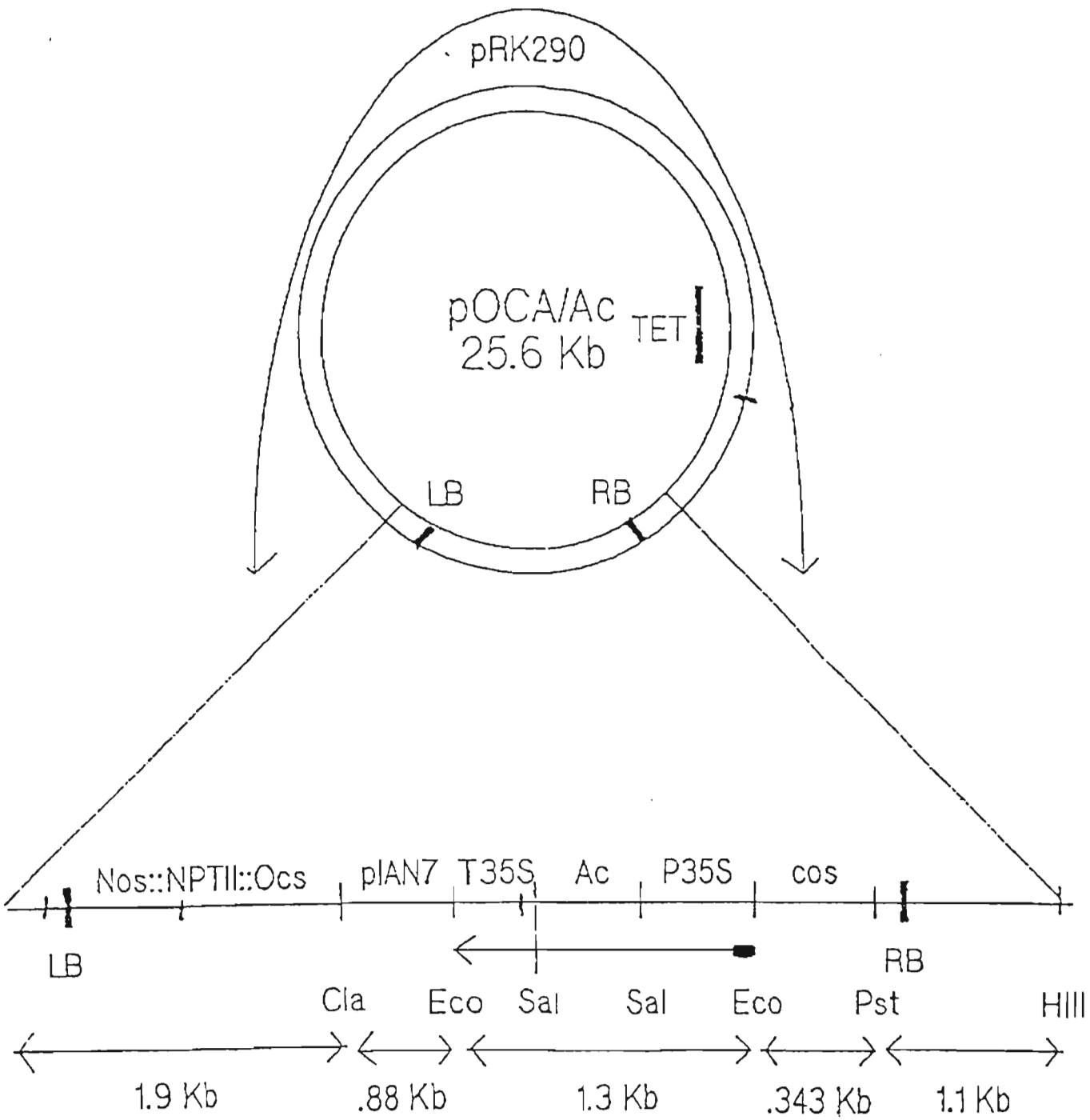
54 g tris
27.5 g boric acid
20 ml 0.5 M EDTA (pH 8)

Made up to 1 litre with distilled water

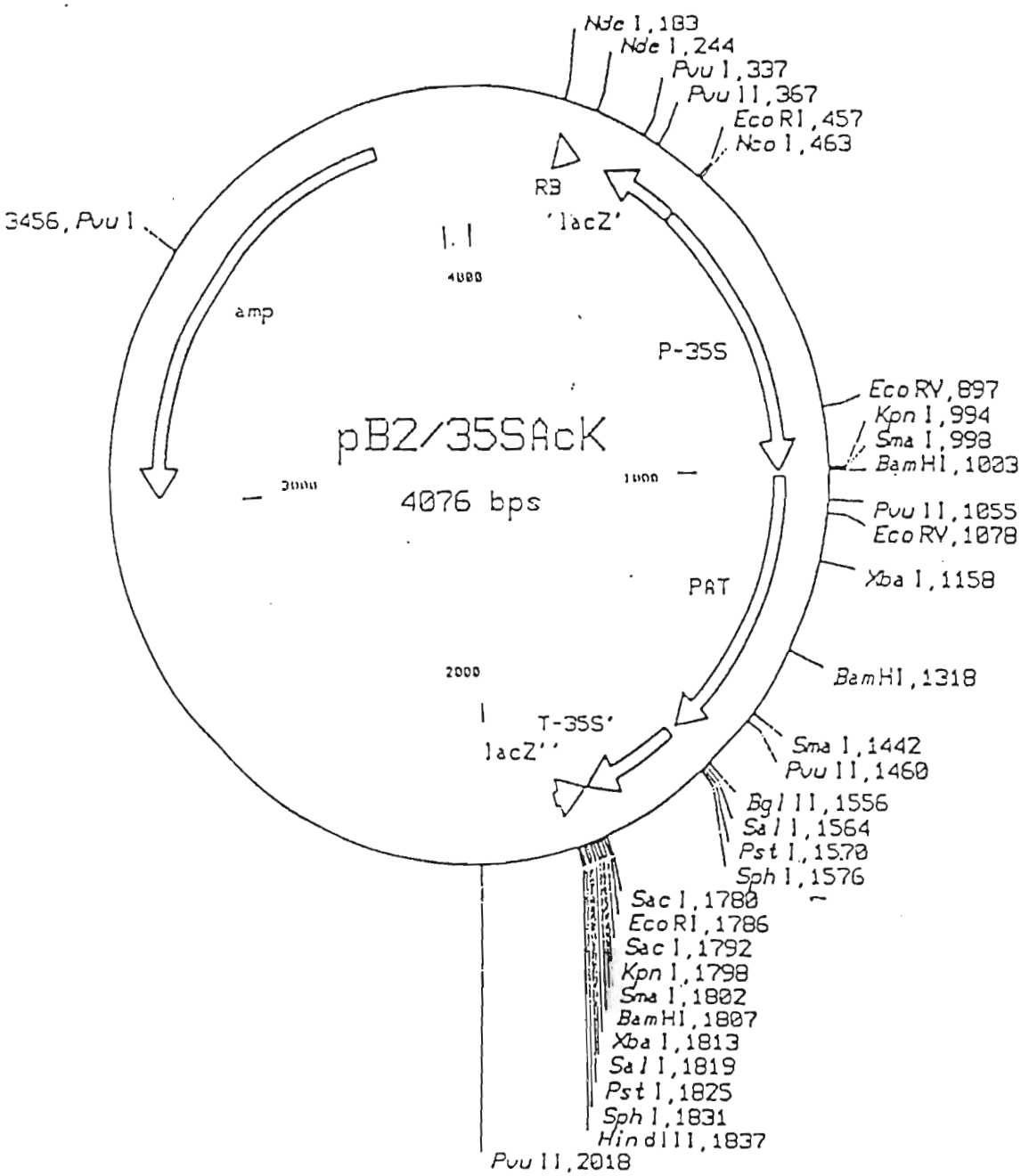
APPENDIX C

PLASMID MAPS

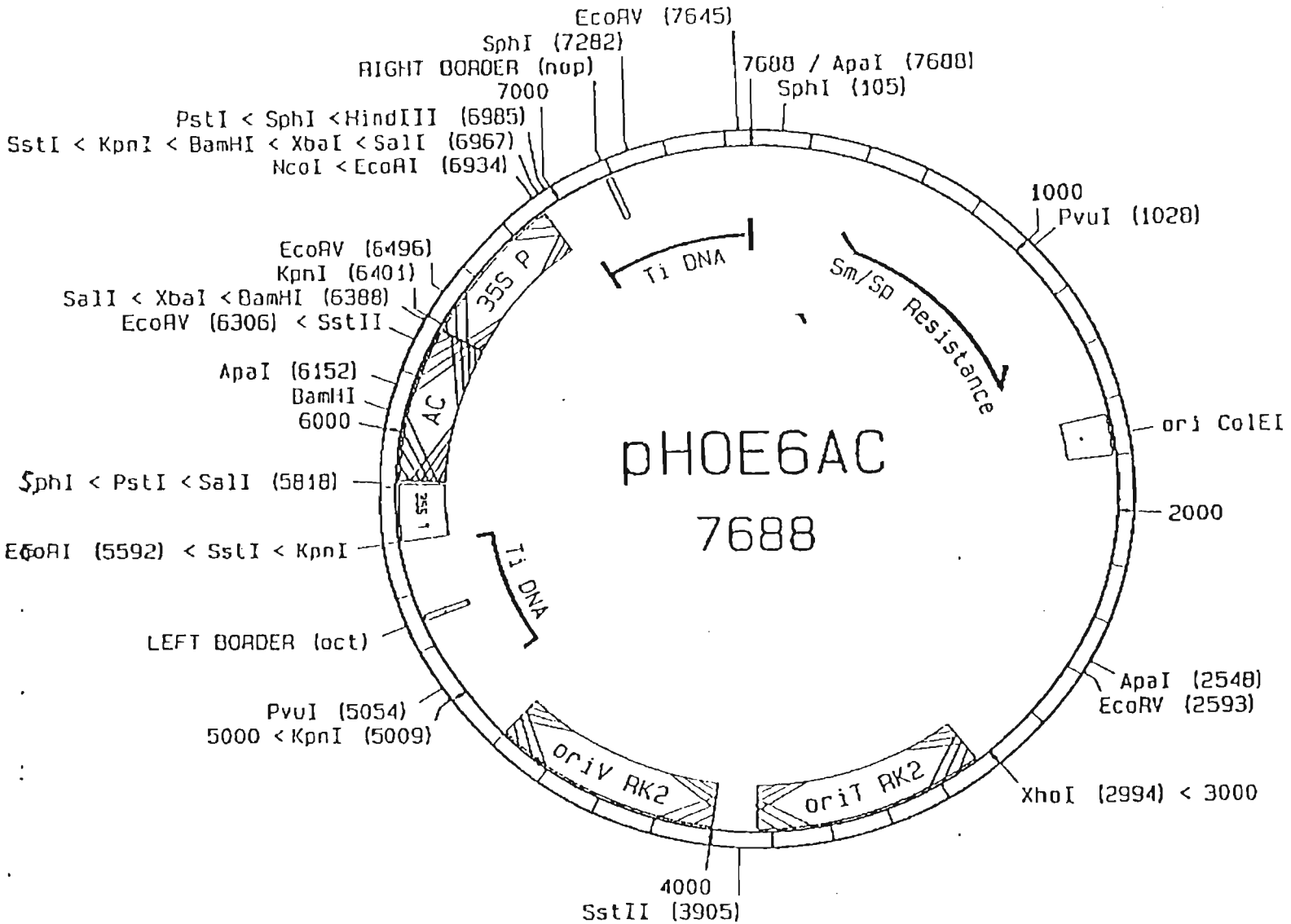
pOCA/Ac



pB2/35SAcK

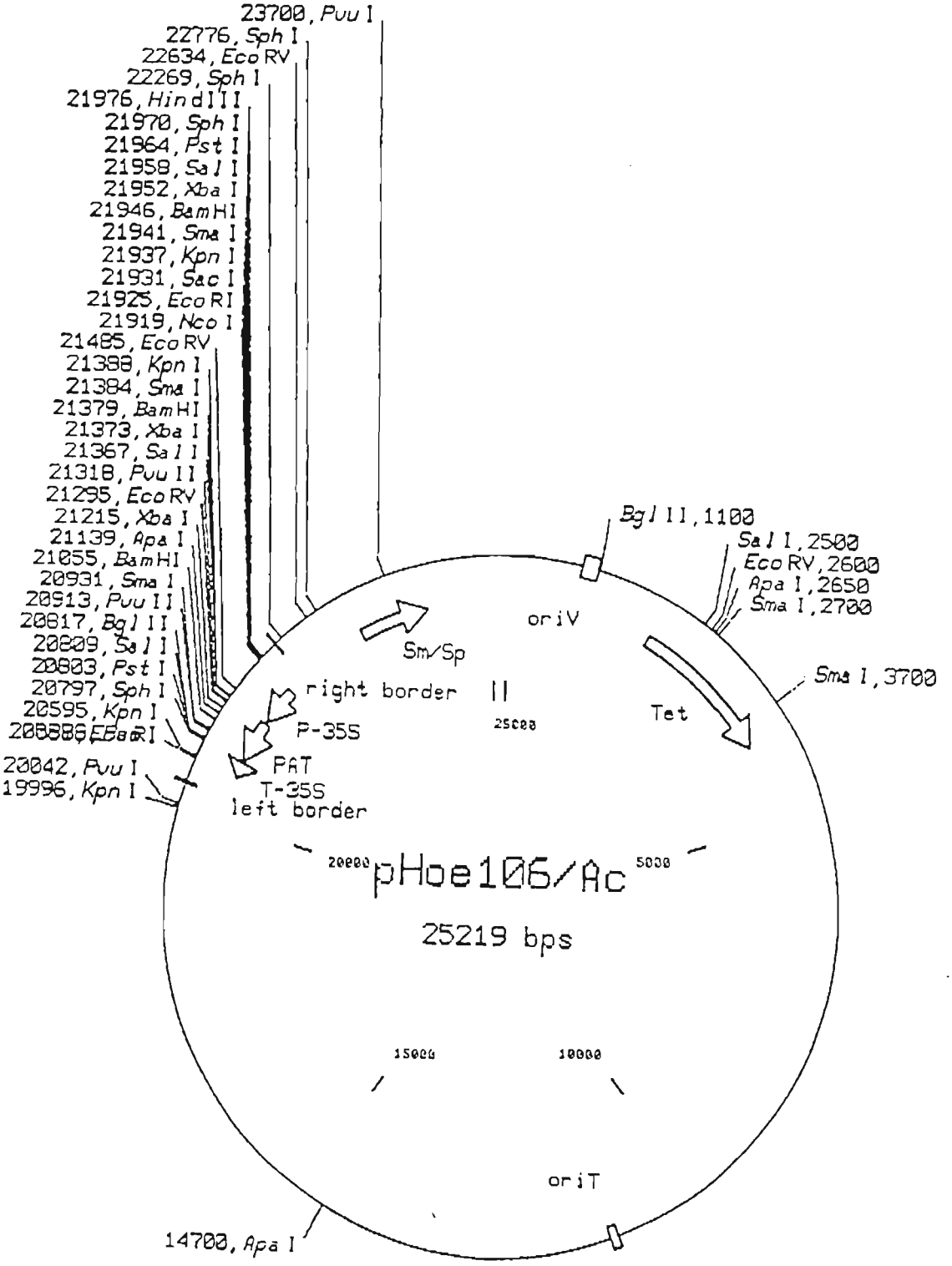

pB2/35SAcK
4076 bps
Nde I, 183
Nde I, 244
Pvu I, 337
Pvu II, 367
EcoRI, 457
Nco I, 463
RB
'lacZ'
3456, Pvu I
amp
P-35S
EcoRV, 897
Kpn I, 994
Sma I, 998
BamHI, 1003
Pvu II, 1055
EcoRV, 1078
Xba I, 1158
PAT
BamHI, 1318
T-35S'
lacZ''
Sma I, 1442
Pvu II, 1460
Bgl II, 1556
Sal I, 1564
Pst I, 1570
Sph I, 1576
Sac I, 1780
EcoRI, 1786
Sac I, 1792
Kpn I, 1798
Sma I, 1802
BamHI, 1807
Xba I, 1813
Sal I, 1819
Pst I, 1825
Sph I, 1831
HindIII, 1837
Pvu II, 2018

pHoe6/Ac

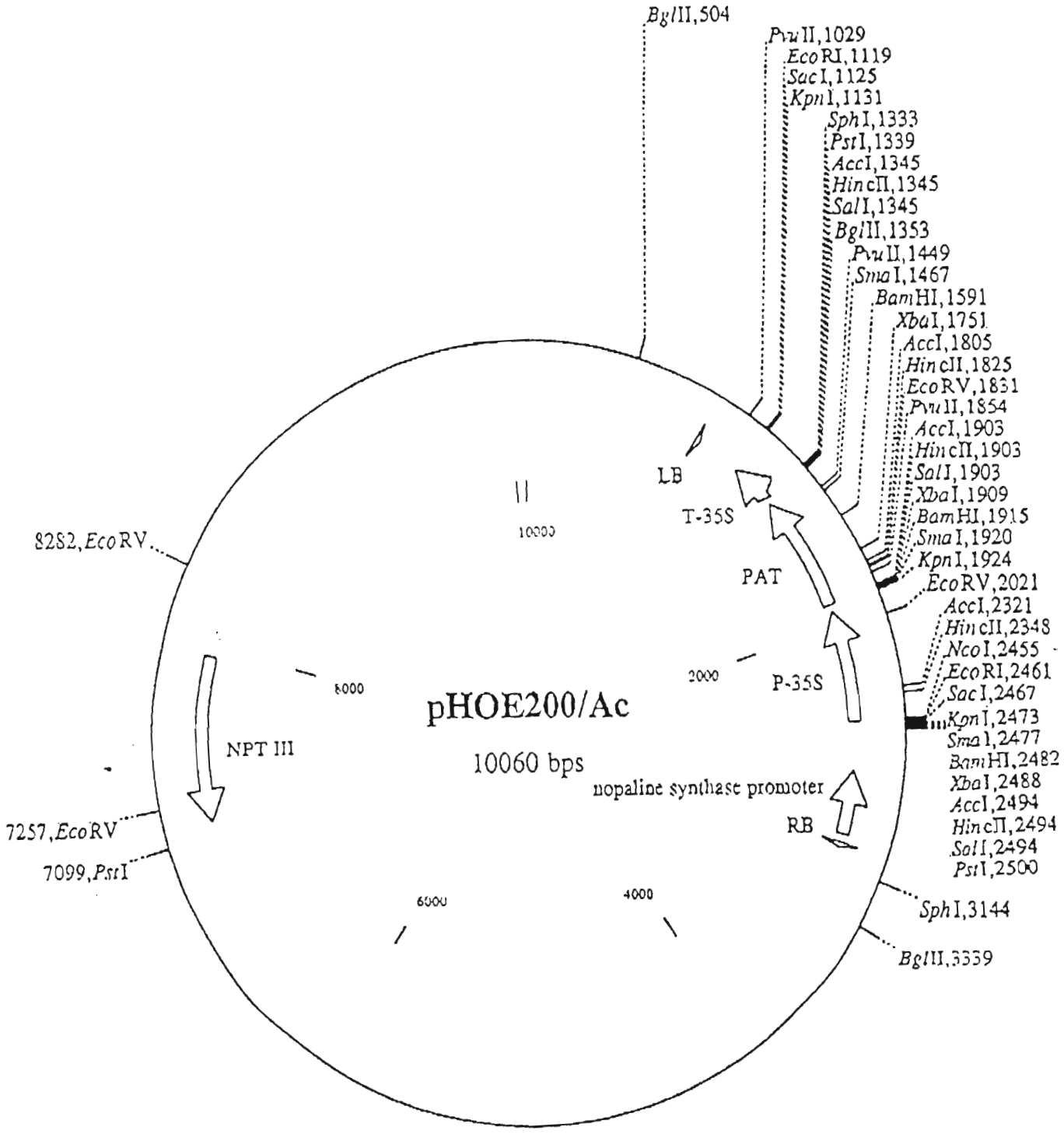


Binary plant transformation vector; can only be used with Agrobacteria, having *trans* acting maintenance and replication functions on genome (e.g. MP90RK, ATHVAK); PPT selection

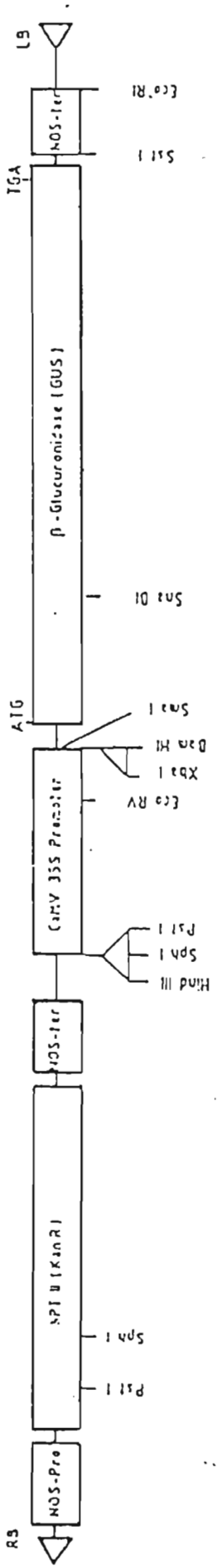
pHoe106/Ac


23700, Puu I
22776, Sph I
22634, Eco RV
22269, Sph I
21976, Hind III
21970, Sph I
21964, Pst I
21958, Sal I
21952, Xba I
21946, BamHI
21941, Sma I
21937, Kpn I
21931, Sac I
21925, Eco RI
21919, Nco I
21485, Eco RV
21388, Kpn I
21384, Sma I
21379, BamHI
21373, Xba I
21367, Sal I
21318, Puu II
21295, Eco RV
21215, Xba I
21139, Apa I
21055, BamHI
20931, Sma I
20913, Puu II
20817, Bgl II
20809, Sal I
20803, Pst I
20797, Sph I
20595, Kpn I
20588, EcoRI
20042, Puu I
19996, Kpn I
14700, Apa I
Bgl II, 1100
Sal I, 2500
Eco RV, 2600
Apa I, 2650
Sma I, 2700
Sma I, 3700
oriV
Sm/Sp
right border
P-35S
PAT
T-35S
left border
Tet
25000
20000
5000
pHoe106/Ac
25219 bps
15000
10000
oriT

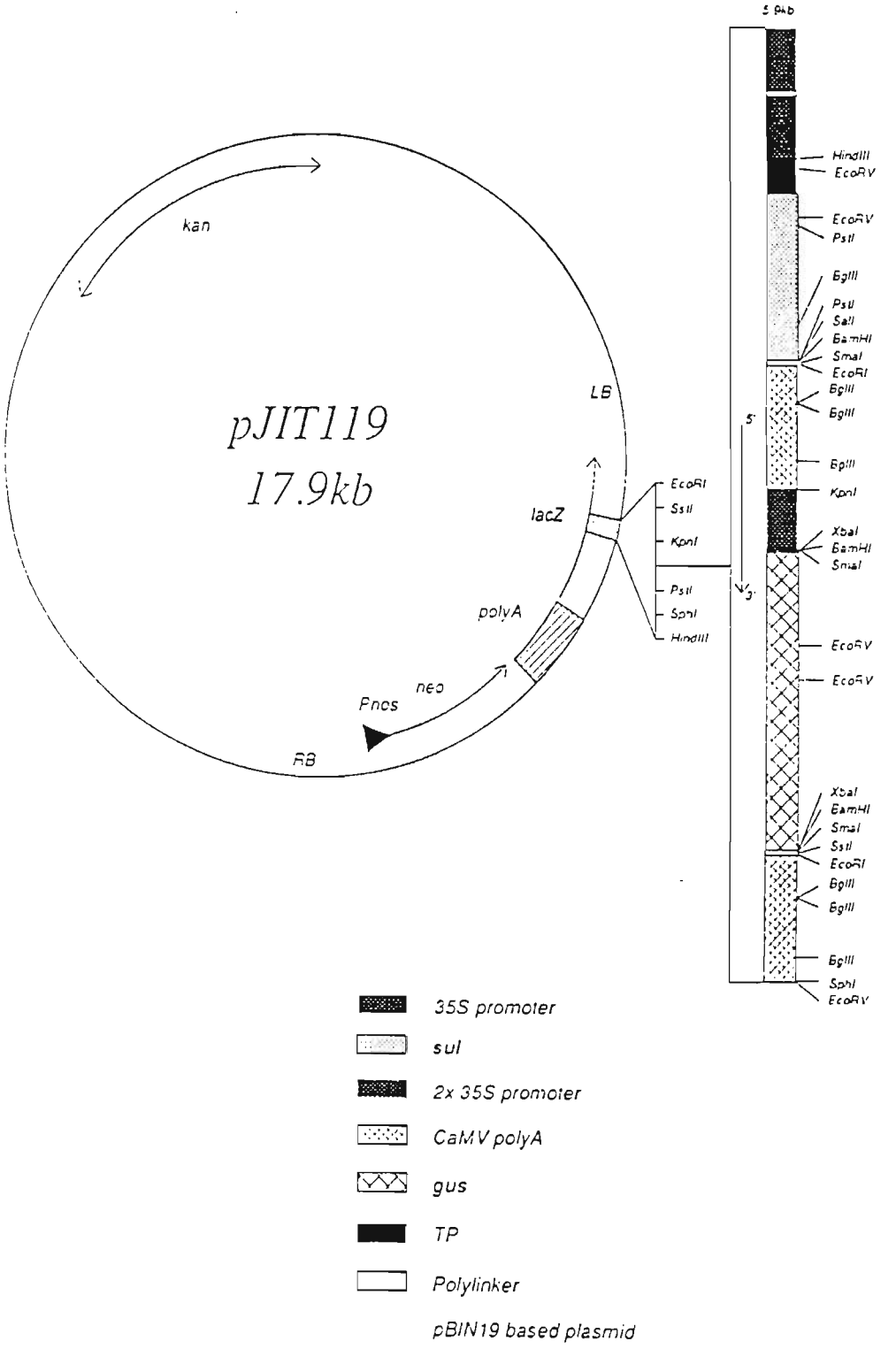
pHoe200/Ac


BglII,504
PvuII,1029
EcoRI,1119
SacI,1125
KpnI,1131
SphI,1333
PstI,1339
AccI,1345
HincII,1345
SalI,1345
BglII,1353
PvuII,1449
SmaI,1467
BamHI,1591
XbaI,1751
AccI,1805
HincII,1825
EcoRV,1831
PvuII,1854
AccI,1903
HincII,1903
SalI,1903
XbaI,1909
BamHI,1915
SmaI,1920
KpnI,1924
EcoRV,2021
AccI,2321
HincII,2348
NcoI,2455
EcoRI,2461
SacI,2467
KpnI,2473
SmaI,2477
BamHI,2482
XbaI,2488
AccI,2494
HincII,2494
SalI,2494
PstI,2500
SphI,3144
BglII,3339
8282,EcoRV
7257,EcoRV
7099,PstI
LB
T-35S
PAT
P-35S
RB
NPT III
pHOE200/Ac
10060 bps
nopaline synthase promoter
10000
2000
4000
6000
8000

pBI121

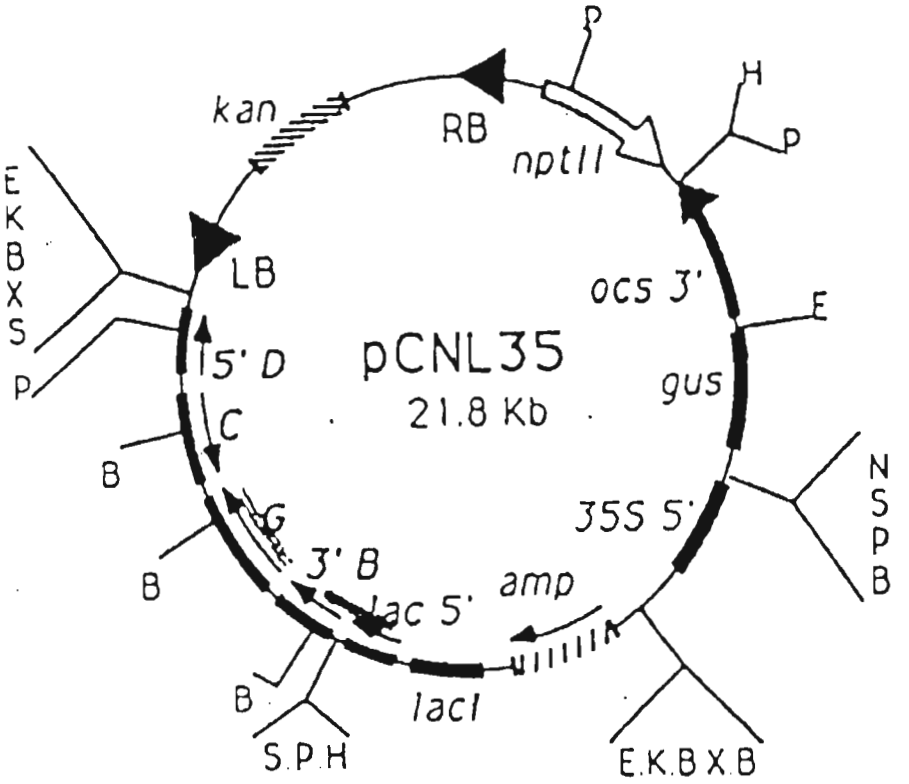

LB
NOS-Ter
Eco RI
Sst I
TGA
β-Glucuronidase (GUS)
Sma I
ATG
Sma I
BamHI
Xba I
Eco RV
CaMV 35S Promoter
Pst I
Sph I
Hind III
NOS-Ter
NPT II (KanR)
Sph I
Pst I
NOS-Pro
RB

pJIT119



Made by J F Guerineau.

pCNL35



Restriction endonuclease sites: B, *Bam* HI; E, *Eco* RI; H, *Hind* III; K, *Kpn* I; N, *Nco* I; P, *Pst* I; S, *Sal* I; X, *Xba* I. RB and LB, right and left borders of pTiT37, respectively. *npt, nos-npt*II-*nos* fusion gene; *lac* 5', promoter of the *lacZYA* operon; *amp, bla* gene for ampicillin resistance; *kan*, gene for kanamycin resistance; *lacI, lac* repressor gene. Inside the circle drawings: B, *vir*B; C, *vir*C; D, *vir*D; G, *vir*G; 3' B, the 3' end of *vir*B; 5' D, the 5' end of *vir*D.

APPENDIX D

ASSAY METHODS

Table A **Summary of minimum inhibitory concentrations of chemical agents used in the selection of transformed soya bean plant material**

Binary vector	Explants	Chemical agent
pJIT119	Cell suspension cultures	100 mg/l kanamycin
pJIT119	Embryonic axes	100 mg/l kanamycin
pJIT119	Cotyledons	300 mg/l kanamycin
pCNL35	Cotyledons	300 mg/l kanamycin
pHoe6/Ac	Cotyledons	80 mg/l glufosinate ammonium
pHoe106/Ac	Cotyledons	80 mg/l glufosinate ammonium
pHoe200/Ac	Cotyledons	80 mg/l glufosinate ammonium
pBI121/Ac	Hypocotyls	20 mg/l kanamycin and 1 mg/l glufosinate ammonium
pBI121/Ac	Cotyledons	20 mg/l kanamycin and 20 mg/l glufosinate ammonium
pBI121/Ac	Shoots	20 mg/l kanamycin and 5 mg/l glufosinate ammonium

Table B **Summary of minimum inhibitory concentrations of chemical agents used in the selection of transformed tobacco plant material**

Binary vector	Explants	Chemical agent
pHoe6/Ac	Leaf discs/shoots	5 mg/l glufosinate ammonium
pHoe106/Ac	Leaf discs/shoots	5 mg/l glufosinate ammonium
pHoe200/Ac	Leaf discs/shoots	5 mg/l glufosinate ammonium
pBI121/Ac	Leaf discs/shoots	20 mg/l kanamycin and 1 mg/l glufosinate ammonium

GUS incubation buffer (Jefferson, 1987)

5 ml 1 M $NaPO_4$ (pH 7)
44 ml sdH_2O
0.25 ml 0.1 M $K_3[Fe(CN)_6]$
0.25 ml 0.1 M $K_4Fe(N_6).3H_2O$
0.5 ml 1 M Na_2EDTA

X-Glu reagent (Jefferson, 1987)

10 mg X-Glu
0.6 ml methanol
2.25 ml GUS incubation buffer

Formyl-aceto-alcohol (FAA) (Sato *et al.*, 1993)

90 ml 50% or 70% ethyl alcohol
5 ml glacial acetic acid
5 ml formalin

Modified plant DNA extraction method (Doyle and Doyle, 1991)

1. Place mortars, pestles and corex tubes at -20°C.
2. Preheat CTAB isolation buffer to 60°C.
3. Grind fresh leaf material to a powder with liquid nitrogen and transfer to corex tubes.
4. Add 5 ml CTAB isolation buffer to each tube.
5. Incubate sample at 60°C for one hour with occasional gentle swirling.
6. Cool to room temperature and extract 1X with an equal volume of chloroform: isoamyl alcohol (24:1) by mixing slowly for five minutes.
7. Centrifuge for ten minutes at 8000 rpm.
8. Transfer top layer into glass tubes and add 2/3 volume of cold 99.9% ethanol. Mix gently to precipitate the nucleic acids (can leave overnight at 4°C).
9. Spool the DNA into sterile eppendorf tubes (can centrifuge at 4000 rpm for five minutes if necessary).
10. Wash DNA with 500 µl wash buffer. Centrifuge at 12 000 rpm for ten minutes.
11. Resuspend the pellet in 100 µl TE and dissolve overnight at 4°C.

CTAB ISOLATION BUFFER: 5% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl(pH 8).

WASH BUFFER: 76% (v/v) ethanol, 10 mM ammonium acetate.

Phosphinothricin acetyltransferase assay (PAT assay) (G. Donn, Personal Communication)

1. Grind approximately 50 mg tissue in liquid nitrogen.
2. Add 50 µl of extraction buffer.
3. Centrifuge at 13 000 rpm for five minutes.
4. Measure protein concentration of the supernatant.
5. Adjust protein concentration to 1 mg/ml with extraction buffer.
6. Take 18 µl supernatant, add 1 µl of 100 mM acetyl-CoA and 1 µl of 10 mM radioactive PPT (alternatively acetyl-CoA can be used as the labelled compound).
7. Incubate at 37°C for one hour.
8. Add 22 µl of 12% TCA (to stop reaction and precipitate protein).
9. Centrifuge at 13 000 rpm for five minutes.
10. Spot 3 x 2 µl of the supernatant on silica thin layer chromotography plates (10 x 20 cm).
11. Develop twice in a solution of 1-propanol:25% ammonia (3:2).
12. Dry tlc plate.
13. Expose to X-ray film overnight.

Note: Tissue extracts must be kept on ice until the reaction is started.
Supernatants may be frozen for later assay.

EXTRACTION BUFFER : 50 mM tris-HCl pH 7.5, 2 mM EDTA

APPENDIX E

SAGENE QUESTIONNAIRE ON IMPORTATION, TRIAL RELEASE OR GENERAL RELEASE OF GENETICALLY MODIFIED ORGANISMS

This questionnaire is a guideline to indicate the scope of information required. Applicants may submit this information in any form, including that in which application has been made to any other statutory body in South Africa or elsewhere.

1. BRIEF DESCRIPTION OF PROPOSED TRIAL RELEASE

2. OBJECTIVE

2.1 What is the aim of the proposed importation/trial release of the genetically modified organism (GMO)? What are the benefits of this approach compared with other possible methods, especially those not involving planned release?

2.2 Should the trial release prove to be successful, is it intended that a general release of the GMO is to be proposed? If so:

2.2.1 When is it proposed that the general release take place?
2.2.2 Where is it proposed that the general release take place?
2.2.3 By whom is it proposed that the GMO be released?

2.3 Is it intended that the GMO be marketed as a product in the Republic of South Africa?

3. NATURE OF ORGANISM AND NOVEL GENETIC MATERIAL

3.1 What is the species of the GMO to be released?

3.2 Is it known whether the unmodified form(s) have any adverse effect on:

3.2.1 humans, animals or plants
3.2.2 agricultural production
3.2.3 any other aspect of the environment

If so, furnish full details of those effects, including any applicable reports.

3.3 Furnish a description of the genetic, and resultant phenotypic, modifications of the GMO. This should include the origin of the inserted DNA, the procedure used to induce the genetic modification and the extent to which it has been characterised.

3.4 What is the frequency of reversion, ie loss of genetic modification?

3.5 What methods are to be used to test for batch to batch consistency?

3.6 On the basis of contained experiments (please describe) indicate:

3.6.1 the survival rates of the GMO in the spectrum of conditions which are likely to be found in the proposed release area(s) and surrounding environment(s);

3.6.2 the capability of the GMO to disperse from the release area and the dispersal mechanisms;

3.6.3 any other relevant information.

(Where reports or publications are available for any of the above information, please furnish copies or references.)

3.7 Should SAGENE at any stage in the future need to ascertain whether the GMO is the same as the GMO specified here how can this be done?

4. TRIAL RELEASE: GENERAL

4.1 Full details are required as to the manner in which the trial release of the GMO is to be undertaken. The following aspects, at least, should be addressed:

4.1.1 the location of the site for the proposed released (eg. ordnance survey map of appropriate scale with site marked)

4.1.2 description of the test site in terms of
* size
* soil
* groundwater level
* topography
* flora and fauna
* climate, especially prevailing winds
* former use
* distance from the nearest human settlements, along with the size of such settlements
* distance from surface waters
* distance from environmentally and otherwise protected areas

4.1.3 description of the environment immediately surrounding the release site

4.1.4 the barriers planned in order to segregate the experiments comprising the trial release from the surrounding environment

4.1.5 the supervision and monitoring of the trial release

4.1.6 the contingency plans to deal with extreme conditions such as storms, floods and bushfires during the course of the trial release;

4.1.7 the provisions to remove or eliminate the GMO from the test site or any other place where it may be found upon completing of the trial release and to restore the test site and any such other place to its *status quo*.

4.1.8 the arrangements for producing the GMO in quantity

4.1.9 the arrangements for transporting the GMO to the release site

4.1.10 the quantity of the GMO to be released.

4.2 What potential hazardous or deleterious effects resulting from the trial release of the GMO can be postulated?

4.2.1 Which of these effects are to be monitored and evaluated during the trial?
4.2.2 How are these effects to be monitored and evaluated during the trial?
4.2.3 If some effects are not going to be monitored, why not?

4.3 Have similar releases of similar GMO's been made before, either within or outside South Africa? If so:

4.3.1 What were the beneficial consequences?
4.3.2 What were the adverse consequences?
4.3.3 What factors might suggest a greater, or a lesser, risk for adverse consequences for the now-proposed trial release?
(Provide references or reports to support your statements.)

4.4 What evidence is there concerning the transferability of the inserted genetic trait to other organisms in the release site and surrounding environment? If transferable:

4.4.1 to which organsisms, and
4.4.2 at what frequencies is it transferable?

4.5 What data are available to suggest that the introduced genetic trait has no deleterious effect upon (the species into which it has been introduced *or* allied species *or* any other organisms *or* the environment in general) in the long term?

4.6 Is the GMO intended to modify the characteristics or abundance of other species? If so, what are :

4.6.1 the target species, and
4.6.2 the intended consequences?

4.7 What experimental results or information are there to show the probable consequences (positive and negative), of the release of such a modified organism, including impacts on:

4.7.1 human, animal or plant health;
4.7.2 agricultural production;
4.7.3 the target and non-target organisms in the area;
4.7.4 the general ecology, environmental quality and pollution in the area; and
4.7.5 genetic resources (eg susceptibility of economically important species to herbicides, pesticides etc)?
What is your assessment of the possible effects?

4.8 Are there any unlikely but possible impacts due to the trial release? If so:

4.8.1 Would any of these have substantial impacts if they actually occurred?
4.8.2 Does the release protocol monitor these low probability risks?

4.8.3 How will these risks be monitored?

4.9 What are the consequences of the organism remaining in the environment beyond the planned period? (Cover the same range of issues as set out in 4.7 and 4.10 above.)

4.10 Has a trial release been carried out in the country of origin of the GMO?

4.10.1 If so, what was the outcome? (Provide documentation from the body controlling the release.)

4.10.2 If not, provide reasons why the trial release was not carried out.

5. TRIAL RELEASE: VACCINES

5.1 For human clinical trials, what arrangements are proposed to dispose of waste containing any vaccine organisms?

5.2 Will the subjects carry live vaccine organisms at the end of the trial? If so,

5.2.1 Will they be likely to disseminate the live vaccine organisms to the general population?

5.3 Based on data obtained in contained experiments (please supply), what are the effects expected when the vaccine organism interacts with target and non-target species in the test area and surrounding environment?

5.4 What is the existing evidence regarding level and duration of immunity produced in the target species?

5.5 What challenge or other tests using virulent field strains are to be carried out on vaccinated animals?

5.6 What is the likelihood that the host vaccine organism would be used in other human or animal vaccines?

5.7 Would the use of this vaccine preclude the future use of the host vaccine organism for immunisation purposes?

6. TRIAL RELEASE: MICROORGANISMS ASSOCIATED WITH PLANTS

6.1 What is the target species of plant?

6.2 Is the organism able to establish itself on/in non-target species in the surrounding environment?

6.3 To what extent does the organism survive and reproduce on/in:

6.3.1 the target plant
6.3.2 the rhizosphere of the target plant species
6.3.3 other plant species in the test site
6.3.4 and surrounding environment?

6.4 What characteristics do you intend to impart to the target plant species?

6.5 Can these characteristics be imparted to non-target plant species, especially those in the surrounding environment? If so:

6.5.1 Is the distribution and abundance of any non-target plant species likely to be affected by the acquisition of these characteristics?

6.6 In the case of soil organisms, what are the effects on organisms likely to be in the test area which are known to be beneficial to plants (eg *Rhizobium*, *Frankia* and mycorrhizal fungi)?

6.7 In the case of soil organisms, what are the effects expected on soil chemistry (eg pH, mineral leaching, chelation, nutrient levels)?

7. TRIAL RELEASE: MICROORGANISMS ASSOCIATED WITH ANIMALS (eg ruminants)

7.1 What is the target species of animal?

7.2 What is known about the organism's ability to survive and reproduce?

7.3 Is the organism able to establish itself in non-target species?

7.4 What characteristics do you intend to impart to the target species of animal (eg ability to degrade pasture toxins)?

7.5 Can these characteristics be imparted to non-target animal species? If so:

7.5.1 Are the distribution and abundance of non-target species likely to be affected by the acquisition of these characteristics?

7.6 In the case of farmed target species, can these characteristics be imparted to feral populations of the target species? If so:

7.6.1 Are the distribution and abundance of such feral populations of the target species likely to be affected by the acquisition of these characteristics?

8. TRIAL RELEASE: MICROORGANISMS TO BE USED FOR MODIFYING THE ENVIRONMENT (eg biological control, pollution control)

8.1 In the case of biological control organisms, what is the biological control target species?

8.2 What direct effects do the unmodified and modified organisms have on:

8.2.1 The target species
8.2.2 Non-target species (including humans)
8.2.3 Any plant or animal species being protected from the target species?