DEVELOPMENT OF HERBICIDE RESISTANCE IN COMMERCIALLY GROWN SOYBEAN AND COTTON CULTIVARS IN SOUTH AFRICA

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2006

D E C L A R A T I O N

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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_____day of _____ 2006

A B S T R A C T

Efficient plant regeneration and transformation procedures and the stability of the transgene are important to the success of the cotton and soybean biotechnology industry. Engineering herbicide resistance into plants will provide the potential solution to effective weed control in agriculture and reduce loss in crop yields due to weeds. It will also provide cheaper control and decrease environmental hazards. The aims of this study were to develop efficient regeneration and transformation protocols for commercially grown soybean and cotton in South Africa and to use tobacco as a model plant to study the stability of the *pat* gene through seed generations, successive generations and high temperature and drought stress regimes.

Tobacco (*Nicotiana tabacum* cv. *Samsun*) leaf disks were successfully transformed with the *pat* gene. PCR analysis confirmed the integration of the *pat* gene in all nine transgenic plants and T1, T2 and T3 progeny. Successive generations, high temperatures and drought stress had no adverse effect on the stability and expression of the *pat* gene in the transgenic tobacco plants.

In view of the economic importance of soybean (*Glycine max*) and cotton (*Gossypium hirsutum*) in South Africa and the potential to improve commercially-grown cultivars by genetic transformation, a regeneration and transformation protocol using the shoot apical meristem and *Agrobacterium*-mediated DNA transfer was successfully developed, to obtain herbicide (Basta) resistant

commercially-grown South African soybean and cotton plants for the first time. The frequencies of regenerated plants per meristem were 66% for Talana, 52% for Ibis, 90% for Sabie, 74.6% for LRCC 101, 69.5% for Palala and 70% for 107/1. Prior to transformation experiments, Talana and Ibis were screened for susceptibility to virulent *Agrobacterium tumefaciens* sis 43. Both cultivars produced tumours in response to infection and were therefore compatible hosts for *Agrobacterium*-mediated DNA transfer. Transformation of Talana, Sabie and 107/1 with the *pat* gene, was successfully achieved following wounding of the shoot apical meristem and injecting with *Agrobacterium* in the presence of acetosyringone. Transformed explants and shoots grew in the presence of kanamycin and PPT, indicating that the integrated *pat* gene was producing the enzyme PAT which was successfully detoxifying the herbicide PPT. Final transformation frequencies from the initial transformed meristems to regenerated plants were 1.06% for Talana, 2.3-3% for Sabie and 1.2-2.3% for 107/1. These transformation frequencies were higher than those reported in the literature. PCR analysis of the extracted DNA from transgenic soybean and cotton shoots confirmed the presence of the 558 bp *pat* coding region in the transformed plants.

The success of this study on the regeneration and transformation of soybean and cotton indicates that South African agriculture now has available techniques for plant regeneration and recombinant DNA technology for crop improvement of soybean and cotton.

In loving memory of my father

Douglas Charles McNaughton

1941-1994

A C K N O W L E D G E M E N T S

I would like to thank my supervisor, Professor M.E.C. Rey, for her advice, encouragement, support and friendship.

Financial support from the University of the Witwatersrand and the Foundation of Research Development is gratefully acknowledged.

Thank you to the Agricultural Research Council in Roodeplaat, Pretoria and Potchefstroom, South Africa, for tobacco plants, virulent *Agrobacterium* cultures and soybean seeds.

Thank you to the Tobacco and Cotton Research Institute, Rustenburg, South Africa, for cotton seeds.

Thank you to Hannah Gerber for her assistance with the statistical analysis.

A very special thank you to my husband, Alex, for his love, support and encouragement, for taking the photographs and assisting with the graphs and tables.

I also want to thank my mom Gwynn and sister Alison for their love and support, and for the many hours spent playing with my daughters, Catherine and Juliette, while I completed the typing of this thesis.

CONTENTS **Page**

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

1.1 Genetic engineering in agriculture

The impressive increases in crop productivity achieved over the last several decades have resulted from genetic improvements in crop cultivars and from advances in agricultural technology and management practices. The non-genetic improvements have included improved weed, disease and insect control through the use of crop protection chemicals, as well as better mechanization, increased supplies of water and nitrogen and optimization of planting densities (Mazur and Falco, 1989; Schmidt, 1995). The introduction of genetic improvements into crops through breeding, a part of agriculture for thousands of years, was more an art than a science until the work of Mendel revealed the rules of inheritance (Gasser and Fraley, 1992). Genetic engineering in agriculture, which in a broad sense refers to any practice that leads to the development of improved cultivars, began with the application of this knowledge (Mazur and Falco, 1989).

With the advent of recombinant DNA technology, with efficient methods of gene cloning, gene isolation, construction and identification, it has become possible to transfer specific and well-characterized traits (genes) across the broadest evolutionary boundaries. As a result, the term "genetic engineering" has been reserved for the isolation, amplification, and *in vitro* manipulation of genes. Refined vector systems, improved selectable markers (for identification of transformants), advances in tissue culture, plant regeneration and transformation methodologies, have enabled the subsequent reintroduction of the genes into living cells or organisms and have enabled transgenic plants to be produced with great efficiency and ease (Gasser and Fraley, 1992).

In 2005, the tenth anniversary of the commercialisation of genetically modified (GM) crops, the 400 millionth hectare of a biotech crop was planted by one of 8.5 million farmers, in one of 21 countries (James, 2005). The 21 countries growing biotech crops in 2005 were, in order of area, the U.S.A., Argentina, Brazil, Canada, China, Paraguay, India, South Africa, Uruguay, Australia, Mexico, Romania, the Philippines, Spain, Colombia, Iran, Honduras, Portugal, Germany, France and the Czech Republic (James, 2005). The global area of approved biotech crops in 2005 was 90 million hectares (ha), equivalent to an annual growth rate of 11% in 2005 (James, 2005). In 2005, the U.S., followed by Argentina, Brazil, Canada and China continued to be the principal adopters of biotech crops globally, with 49.8 million ha planted in the U.S. (55% of global biotech area) of which approximately 20% were stacked products containing two or three genes, with the first triple gene product making its debut in maize in the U.S (James, 2005). The largest increase in any country in 2005 was in Brazil, provisionally estimated at 4.4 million ha, followed by the U.S. (2.2 million ha), Argentina (0.9 million ha) and India (0.8 million ha) (James, 2005).

Biotech soybean continued to be the principal biotech crop in 2005, occupying 54.4 million ha (60% of global biotech area), followed by maize (21.2 million ha at 24%), cotton (9.8 million ha at 11%) and canola (4.6 million ha at 5% of global biotech crop area) (James, 2005). During the first decade herbicide tolerance has consistently been the dominant trait followed by insect resistance and stacked genes for two traits. As a result of biotech crops the accumulative reduction in pesticides for the period 1996 to 2004 was estimated at 172, 500 MT of active

ingredient, which is equivalent to a 14% reduction in the associated environmental impact of pesticide use on these crops (James, 2005).

Genetic traits have been introduced into modern cultivars that improve their insect resistance (Gasser and Fraley, 1992; Pannetier *et al*., 1997), disease resistance (Staskawicz *et al*., 1995; Tricoli *et al*., 1995; Di *et al*., 1996; Chen and Punja, 2002; Sanjaya *et al*., 2005; Tohidfar *et al*., 2005; Valenzuela *et al*., 2005), herbicide resistance (Shah *et al*., 1986; D'Halluin *et al*., 1990; Gasser and Fraley, 1992; Zhou *et al*., 1995; Park *et al*., 1996; Clemente *et al*., 2000; Chen and Punja, 2002; Choi *et al*., 2003; Goldman *et al*., 2003; Manickavasagam *et al*., 2004; Shu *et al*., 2005), harvesting and processing qualities (Gasser and Fraley, 1992; Hiatt, 1993), adaptation to particular environmental conditions (Finnegan and McElroy, 1994; Sivamani *et al*., 2000; Han *et al*., 2005), yield (through traits such as heterosis and lodging resistance) and nutritional qualities (through the development of low-glucosinilate and low-erucic acid lines of oilseed rape) (Uchimaya *et al*., 1989; Gibbons, 1990; Yoder and Goldsbrough, 1994; Schmidt, 1995).

There are significant agronomic, health and environmental benefits associated with genetically modified crops (Carpenter and Gianessi, 2000; James, 2005). These include the following: (1) more sustainable and resource-efficient crop management practices that require less energy/fuel and conserve natural resources; (2) more effective control of insect pests and weeds- a reduction in the overall amount of pesticides and herbicides (Carpenter and Gianessi, 2000) used in crop production, which impacts positively on bio-diversity, protects predators

and non-target organisms, and contributes to a safer environment; (3) less dependency on conventional insecticides and herbicides that can be a health hazard to producers and consumers; (4) greater operational flexibility in timing of herbicide and insect applications; (5) conservation of soil moisture, structure, nutrients and control of soil erosion through no-or-low tillage practices as well as improved quality of ground and surface water with less pesticide and herbicide applications. There is an increasing body of evidence that genetically modified crops in conjunction with conventional practices offer a safe and effective technology that can contribute to a better environment and more sustainable and productive agriculture (Carpenter and Gianessi, 2000; James, 2005).

Carpenter and Gianessi (2000) reported on the advantages of the introduction of Roundup Ready crops and herbicide use. According to these authors, although the total amount of the herbicide Glyphosate used with soybeans has changed little with the introduction of Roundup Ready varieties, the data show a substantial reduction in the number of applications made to soybean acreage. This demonstrates growers using fewer active ingredients and making fewer trips over the field, which translates into ease of management and cost savings. The primary reason growers have adapted Roundup Ready weed control programmes is the simplicity of a weed control programme that relies on one herbicide to control a broad spectrum of weeds without crop injury or crop rotation restrictions. As for economic benefits, the introduction of Roundup Ready varieties has provided an overall saving in herbicide costs for both adopters and non-adopters of the technology.

The exploitation of transformed plants for studies on gene regulation and developmental biology is in progress; newly inserted foreign genes have been shown to be stably maintained during plant regeneration and are transmitted to progeny as typical Mendelian traits (Satyavathi *et al*., 2002). Plant transformation will clearly be a powerful and broadly applicable methodology that will make crop breeding faster, more predictable and more far-reaching. The range of plant species transformed is continually being extended (Chen and Punja, 2002; Bregitzer and Tonks, 2003; Hoshi *et al*., 2004). Soybean and cotton have fallen into this category. The application of molecular biology techniques, including genetic engineering, has resulted in significant gains made for many agricultural crops, some of which are already part of commercial applications (Kasha, 1999).

The production of transgenic plants can be divided into four main stages (Rogers *et al*., 1986): (1) introduction of foreign genes into plasmids and modified *Agrobacterium tumefaciens* strains, (2) introduction of foreign genes into plant cells or tissues using DNA transfer procedures, (3) selection and regeneration of transformants and (4) analysis and verification of gene expression in transformed plants.

Proof of integrative transformation requires: (1) controls for treatment and analysis, (2) a tight correlation between treatment and predicted results, (3) a tight correlation between physical (Southern blot) and phenotypic (enzyme assay) data, (4) Southern analysis, (5) data that allows discrimination between false positives and correct transformants in the evaluation of the phenotypic evidence, (6) correlation of the physical and phenotypic evidence with transmission to sexual

offspring and (7) molecular and genetic analysis of offspring populations (Potrykus, 1991; Hiatt, 1993; Zhou *et al*., 1995).

The basic steps in the development of a transformation system for explants is as follows (Draper *et al*., 1988): (1) determining a suitable medium which encourages regeneration of a wide range of explants, (2) defining the most favourable environmental conditions for donor plant growth in order to provide the most responsive explants, (3) evaluating the best type and size of explant to use with a particular volume of medium in a specific culture vessel, (4) determining the levels of a suitable antibacterial agent (cefotaxime or carbenicillin) which inhibits growth of *Agrobacterium* but still allows reasonably normal callus formation and shoot regeneration (when *Agrobacterium*-mediated DNA transfer is used), (5) determining the minimum levels of selection agents (antibiotics such as kanamycin or hygromycin) which inhibit callus formation, shoot regeneration and rooting of shoots, (6) developing inoculation and incubation conditions which enable the explant to survive exposure to *Agrobacterium* for sufficient time to allow transformation to take place without interfering with callus or shoot regeneration (when *Agrobacterium*-mediated DNA transfer is used) and (7) determining the optimum combination of *vir* helper system, Ti plasmid and *Agrobacterium* chromosomal background (when *Agrobacterium*-mediated DNA transfer is used).

1.2 Genetically modified crops in South Africa

Genetically modified crops have been commercially grown in South Africa since 1997 when *Bacillus thuringiensis* (*Bt*) resistant cotton was introduced (http://www.monsanto.co.za). In 2004, South Africa planted an estimated 500 000 ha of GM crops (http://www.monsanto.co.za). This makes South Africa the seventh largest producer of GM crops in the world (http://www.monsanto.co.za). GM maize, soybeans and cotton are planted around the country, with field trials on canola, sugar cane, potato and strawberry (http://www.monsanto.co.za). The planting of GM crops in South Africa is the first in Africa and a significant development (http://www.monsanto.co.za). Most commercially available GM seed varieties are based on a small number of genetically modified organisms that the patent holder licences seed companies to use on their own seed. Seed companies in South Africa purchase the rights to use the genetically modified organisms to cross with their own hybrids (http://www.monsanto.co.za). All GM seed imported into South Africa contains Monsanto technology (http://www.monsanto.co.za). Between 2001 to March 2005, 15.3 tons of GM soybean seed (GTS 40-3-2), more than 10 tons of GM cotton and more than 6 tons of GM maize was imported into South Africa for trials or commercial planting (http://www.monsanto.co.za).

In 2003, an estimated 15-20% of all maize grown in South Africa was genetically modified (http://www.monsanto.co.za). Most GM maize planted is insect resistant (*Bt*), with herbicide tolerant maize (Roundup Ready) only released for commercial use in 2003 (http://www.monsanto.co.za).

Cotton is not a major sector in South Africa, planted only in a few fertile areas of the country. As a result, South Africa does not produce enough cotton for domestic needs and has to import the shortfall each year. A number of cotton events have been approved for commercial use or trial in South Africa. These are all based either on *Bt* events or herbicide tolerance. *Bt* cotton includes a number of lines of Monsanto's Bollgard (budworm and bollworm resistance) and Bollgard ll cotton (same as Bollgard plus resistance to secondary leidopteran pests), RR1445 (Roundup Ready) commercialised in 2001, MON88913 (glyphosate tolerance) and BXN (bromoxymil tolerance, developed by Calgene) (http://www.monsanto.co.za). Adoption of GM cotton varieties has been rapid in South Africa. *Bt* cotton had an estimated 35% of the cottonseed market in 2003, cotton with stacked GM traits had a 30% share of the market and Roundup Ready cotton had a 10% share-giving all types of GM cotton a 75% of total cottonseed planted in that year (http://www.monsanto.co.za). Cotton was grown on 36 000 ha in 2003 (http://www.monsanto.co.za). Over 90% of small-scale farmers grow GM cotton. *Bt* cotton has been planted by small-scale farmers in the Makhathini Flats on the northeast coast of South Africa (KwaZulu-Natal Province). The yields and profits from the GM cotton crops for small-scale farmers in South Africa have been boosted. A study of cotton grown by more than 2,000 farmers showed that those that planted *Bt* cotton benefited by US$86 to US$93 per hectare more than those that planted conventional strains (Scott, 2004). To cotton farmers, growing *Bt* cotton would mean spending less money and time on spraying crops with pesticides. These results could be significant for Africa's agricultural economy: cotton is grown on 2.5 million ha of the continent, most of

it on small plots of less than 10 ha (Scott, 2004). Cotton production areas in South Africa are the Northern Cape-Lower Orange River and Griqualand West, North West-Rustenberg, Northern Province- Limpopo Valley, Loskop and Springbok Flats, Mpumalanga and KwaZulu-Natal (http://www.cottonsa.org.za).

Like cotton, the soybean industry in South Africa is small. However, unlike cotton, it is a growing industry. Only one soybean event, GTS40-3-2 (glyphosate tolerance), has been approved in South Africa. It was commercialised in 2001 and Monsanto holds the patent (http://www.monsanto.co.za). Genetic modification of soybeans is for herbicide (glyphosate) tolerance (Roundup Ready), and the market share of GM soybeans was estimated at between 22% and 30% in 2004 (Feris, 2004). Even at the upper limit, this would be 41 000 ha under GM soybeans for 2004 (http://www.monsanto.co.za). Various soybean cultivars are very well adapted to South African conditions. Soybeans are mainly cultivated under dryland conditions and grown mainly in Mpumalanga and KwaZulu-Natal, while small quantities are also cultivated in the Free State, Northern Province, Gauteng and North West Province (http://www.nda.agric.za). Modified soybean, used mostly for the production of animal and poultry feed, was planted on 750 ha of land in Mpumalanga (Cook, 2002).

1.3 Herbicides in agriculture

Even people whose experience with weeds has been limited to hand cultivating a home garden can appreciate the usefulness and efficiency herbicides provide for commercial agriculture. Herbicide treatments are an integral part of modern agriculture because they provide cost-effective increases in agricultural

productivity (Marshall, 1998). Increased yields result from reduced weed competition for water, light and nutrients (De Block *et al*., 1987; Mazur and Falco, 1989; Gasser and Fraley, 1992). In addition, crop quality often improves in the absence of contaminating weed seeds, such as wild mustard seeds in harvested canola or wild garlic seeds in wheat. Herbicides can also aid soil conservation efforts through no-till agricultural practices, wherein herbicides rather than tillage are used to reduce weed populations prior to planting (Mazur and Falco, 1989).

Several classes of herbicides can be effective for broad-spectrum weed control, but these are either non-selective, killing also the crop plants, or they significantly injure some crops at the application rates required (Fox, 1990; Dekker and Duke, 1995). Additionally, often the most desirable herbicides for weed control and crop safety have other less desirable characteristics (e.g. environmental and economic) (Dekker and Duke, 1995).

Herbicides have traditionally been discovered by screening novel compounds in a series of increasingly specific tests (Mazur and Falco, 1989). Compounds are first tested for activity against a spectrum of weeds and lack of activity against crops (Mazur and Falco, 1989). Promising compounds are further tested in more extensive greenhouse screens and finally in small-scale field trials (Mazur and Falco, 1989). The herbicides must then run the gauntlet of acute and chronic toxicology tests (Mazur and Falco, 1989).

To be commercially successful, herbicides must have potent biological activity against a broad spectrum of weeds and at the same time be non-toxic to crop

plants, mammals and invertebrates, have relatively short soil residual properties and have favourable production costs (de Greef *et al*., 1989). In the 1950s about 1 in 2000 screened compounds were commercialized and by the 1970s, the rate had dropped to approximately 1 in 7 000 compounds, while in the 1980s hardly 1 in 20 000 compounds emerged from these screens (Mazur and Falco, 1989).

Selective toxicity of herbicides to weeds but not to crops is one of the most difficult properties to achieve, as might be expected from the biological relatedness of weeds and crops. Selectivity is a function of the physico-chemical properties of a compound, and of the biochemical interactions of the compound with the crop and the weeds. For example, herbicides that do not percolate beyond the top soil layer, and thus do not affect crop roots that extend below this layer, can provide selectivity. Environmental conditions such as climate, soil pH and soil organic content influence these interactions. For some compounds, management practices (e.g. timing and/or site of the application) can be used to impart selectivity. Glyphosate, a non-selective herbicide, is used prior to planting as a substitute for tillage, thereby taking advantage of its rapid and wide-spectrum weed killing activity and short lifetime in the soil (Mazur and Falco, 1989).

A number of important classes of herbicides (e.g. the triazines, sulfonylureas, and imidazolinines) are more toxic to weeds than to specific crops. In these examples, selectivity results from a unique or enhanced metabolic detoxification of the herbicide by the crop plant but not by the weed. In other cases, herbicide selectivity results from the sequestering of the herbicide within an internal compartment of the crop plant. Alternatively, external barriers such as plant

cuticles can prevent penetration of the herbicide. In some cases it has been possible to achieve selectivity by seed coat applications of a "safener", a second chemical that reduces the toxicity of the herbicide to the crop (Mazur and Falco, 1989).

Engineering herbicide tolerance into crops represents an alternative for conferring selectivity of herbicides and enhancing crop safety. Genetic modification of crops to make them herbicide resistant could remove a major factor in determining the choice of herbicides available for use by farmers. It could allow for the wider use of more effective herbicides with broader weed-control spectra. In addition, compounds that are effective at low application rates, have short lifetimes in the soil, and have more favourable toxicological properties, might become more generally useful if the constraint of crop selectivity were removed (Fox, 1990). These possibilities would extend beyond major acreage crops to minor acreage crops that have not yet benefited from effective weed control compounds (D'Halluin *et al*., 1992). One disadvantage, however, would be market domination by a few herbicide companies.

Genetic modification could also complement and enhance existing herbicide selectivity by increasing the margin of safety for selective compounds, particularly during periods of environmental stress when plant metabolism is reduced (Gasser and Fraley, 1992; Marshall, 1998). It could also allow the grower to increase the application rates for selective herbicides. The introduction of herbicide resistance into crops could give the grower greater flexibility in choosing crops for rotations or double crop plantings (such choices are currently

limited by the differential sensitivities of crops to particular herbicides). The development of crop plants that are tolerant to herbicides with low toxicity, low soil mobility, rapid biodegradations and with broad spectrum activity against various weeds would provide more effective, less costly and more environmentally attractive weed control. The commercial strategy in engineering herbicide tolerance is to gain market share through a shift in herbicide use, not to increase the overall use of herbicides. Herbicide resistant plants will have the positive impact of reducing overall herbicide use through substitution of more effective and environmentally acceptable products (Gasser and Fraley, 1992; Marshall, 1998). Thus the combination of crop protection chemical technology and genetic technology will provide a new range of management options for more effective weed control (Mazur and Falco, 1989; Marshall, 1998). Herbicide resistant plants could play a key role in an integrated plant protection scheme (van der Hoeven *et al*., 1994).

Two general approaches have been taken in engineering herbicide resistance or tolerance: 1) altering the level and sensitivity of the target enzyme for the herbicide (Stalker *et al*., 1988; Gasser and Fraley, 1992), and 2) incorporating a gene that will detoxify the herbicide before it reaches the biochemical target inside the plant cell (Stalker *et al*., 1988; Gasser and Fraley, 1992). As an example of the first approach, canola and soybean have been made tolerant to Roundup herbicide by introducing DNA coding for the overproduction of herbicide-resistant analogs of 5-enolpyruvylshikimate-3-phosphate synthase, the target of Roundup activity (Stalker *et al*., 1988; Gasser and Fraley, 1992). Similarly, resistance to sulfonylurea compounds, the active ingredient in Glean

and Oust herbicides, was conferred by introducing mutant acetolactate synthase genes into canola, cotton and tobacco (Lee *et al*., 1988; Fraley, 1992).

Resistance to glufosinate, the active ingredient in Basta and bromoxynil has been conferred by the alternative approach of introducing bacterial genes encoding enzymes that inactivate the herbicides by acetylation or nitrile hydrolysis, respectively (Fraley, 1992). Glufosinate-tolerant canola, maize and bromoxynil-tolerant cotton have proved themselves in field trials (Fraley, 1992).

In recent years, a major effort has been made in several laboratories to engineer herbicide resistant plants. The development of herbicide-resistant crop plants was one of the first commercial applications of plant genetic engineering (Freyssinet and Cole, 1999). This research has yielded promising results in the case of resistance to glyphosate (Comai *et al*., 1985; Shah *et al*., 1986; Mazur and Falco, 1989; Padgette *et al*., 1995; Penaloza-Vazquez *et al*., 1995; Schmidt, 1995; Zhou *et al*., 1995; Clemente *et al*., 2000), bromoxynil (Stalker *et al*., 1988), the sulfonylurea and imidazolinone herbicides (de Greef *et al*., 1989) and phosphinothricin and glufosinate (De Block *et al*., 1987; D'Halluin *et al*., 1992; Zhou *et al*., 1995; Park *et al*., 1996; McCabe *et al*., 1999; Zhang *et al*., 1999a; Sarria *et al*., 2000; Chen and Punja, 2002; Choi *et al*., 2003; Goldman *et al*., 2003; Leibbrandt and Snyman, 2003; Manickavasagam *et al*., 2004; Zeng *et al*., 2004; Shu *et al*., 2005). Commercial application of herbicide resistance in the field has been reported for tobacco and potato (de Greef *et al*., 1989), sugarbeet (Buckman *et al*., 2000), rice (Jiang *et al*., 2000), soybean (Somers *et al*., 2003) and cotton (May *et al*., 2004).

1.4 The herbicide Basta

Glufosinate ammonium is the active ingredient of the herbicide Basta (Hoechst, AG, Frankfurt/Main, FRG) and is a broad spectrum, non-selective contact herbicide of widespread use throughout agriculture. Glufosinate ammonium is the synthetic form of the herbicide produced as phosphinothricin (PPT). The uptake of PPT is restricted to leaves and roots of plants; woody parts are not affected (Droge *et al*., 1992), therefore the use of this non-selective herbicide is limited to specific applications, for example in vineyards and in fruit orchards. Genetically engineered herbicide resistant plants have extended the agricultural application of Basta (Droge *et al*., 1992). Its utility as a herbicide stems from the fact that it is non-toxic to mammals (Donn, 1991), is rapidly degraded by soil organisms (Donn, 1991) and effectively controls broadleaf and grassweeds, sisal, reeds, bulrushes and greenwattle (Donn, 1991). Damage symptoms in the form of yellowing and leaf scorch to the aerial portions of the plants commence seven to ten days after treatment and control is normally observed approximately fourteen days after spraying (Donn, 1991).

PPT (an analogue of glutamic acid produced by *Streptomyces viridochromogenes*) inhibits the plant amino acid enzyme glutamine synthetase (GS) (Bayer *et al*., 1972). GS plays a central role in the assimilation and detoxification of ammonia produced by nitrate reduction and nitrogen fixation in roots, as well as for the re-assimilation of ammonia released by photorespiration in leaves, and in the regulation of nitrogen metabolism in plants (Donn *et al*., 1984; Eckes *et al*., 1989; Downs *et al*., 1994). In plants, GS is the only enzyme that can detoxify the

ammonia released by nitrate reduction, amino acid degradation and photorespiration (Eckes *et al*., 1989). Inhibition of GS by PPT leads to a rapid accumulation of intracellular ammonia levels and an associated disruption of chloroplast structure, resulting in the inhibition of photosynthesis and eventual cell death (Donn *et al*., 1984; Tashibana *et al*., 1986; De Block *et al*., 1987; Eckes *et al*., 1989; Downs *et al*., 1994).

Highly efficient PPT resistant genes have been isolated from two different *Streptomyces* species: the phosphinothricin acetyltransferase (*pat*) gene from *Streptomyces* (*S*.) *viridochromogenes* Tu494 (Bayer *et al*., 1972; Wohlleben *et al*., 1988; Alijah *et al*., 1991) and the bialaphos resistance (*bar*) gene from *S. hygroscopicus* ATCC21705 (Murakami *et al*., 1986; Botterman and Leemans, 1988; de Greef *et al*., 1989; D'Halluin *et al*., 1992; van der Hoeven *et al*., 1994). The *bar* gene shows significant sequence homology to the *pat* gene (Donn, 1991). Both these genes produce the enzyme phosphinothricin acetyltransferase (PAT) which acetylates the free ammonia group of PPT thereby detoxifying it (Murakami *et al*., 1986, De Block *et al*., 1987; Thompson *et al*., 1987). These producer strains possess highly sensitive glutamine synthetases, but show no signs of self-toxification (Bayer *et al*., 1972).

A third and distinctive gene, based on the *pat* gene, has been synthesized by Hoechst and been used by various researchers for transfer experiments (Donn, 1991). The *pat* and *bar* genes contain 70 % Guanine(G) Cytosine(C)-pairs in their sequence, while typical plant genes have only 50 % GC-pairs. Cytosine in the DNA are targets for methylation enzymes. DNA methylation is one

mechanism with which organisms can inactivate genes by suppressing its expression. Due to its high GC content, Hoechst feared that plant cells could inactivate the *Streptomyces* gene. They therefore decided to design a synthetic version of the *pat* gene with a lower GC content (Donn, 1991). Researchers have genetically manipulated this *pat* gene so that it could be transformed into a wide range of dicotyledonous plant species (Zeng *et al*., 2004).

Several approaches have been used to genetically engineer plants that were resistant to the non-selective herbicide Basta. Transgenic plants have been produced based on mutant forms of the target enzyme for the herbicide (Botterman and Leemans, 1988; D'Halluin *et al*., 1992) and plants that expressed the *pat* or *bar* gene that coded for the PAT enzyme. Transgenic PPT resistant plants expressing the *pat* or *bar* gene include tobacco (De Block *et al*., 1987; Wohlleben *et al*., 1988; van der Hoeven *et al*., 1994), potato (De Block *et al*., 1987), alfalfa plants (D'Halluin *et al*., 1990), the herb *Scoparia dulcis* (Yamazaki *et al*., 1996), papaya (Cabrera-Ponce *et al*., 1995), rice (Cao *et al*., 1992; Rathore *et al*., 1993; Chen *et al*., 1998), sugarbeet (D'Halluin *et al*., 1992), rye (Castillo *et al*., 1994), lettuce (McCabe *et al*., 1999), conifers (Bishop-Hurley *et al*., 2001), pearl millet (Goldman *et al*., 2003), carrots (Chen and Punja, 2002), cotton (http://www.ogtr.gov.au), cassava (Sarria *et al*., 2000), oat and barley (Zhang *et al*., 1999b; Bregitzer and Tonks, 2003), soybean (Zeng *et al*., 2004), sugar cane (Leibbrandt and Snyman, 2003; Manickavasagam *et al*., 2004) and perennial grass (Shu *et al*., 2005). Some of these plants have been tested under greenhouse and field conditions and found to be resistant to field doses of commercially available PPT. Progeny analysis has shown that the transgenic trait is inherited by the

progeny (Rathore *et al*., 1993; Yamazaki *et al*., 1996; Chen *at al*., 1998; Zhang *et al*., 1999a, Zeng *et al*., 2004; Shu *et al*., 2005). Genetic engineering of PPT tolerance into South African commercially grown soybean and cotton is of significant interest and would be beneficial to agriculture.

1.5 Gene transfer to plants

Recent advances in the production of transgenic plants have resulted from efficient systems for introducing foreign genes into plants (Horsch *et al*., 1985; Rogers *et al*., 1986). Efficient methods are important for understanding and controlling plant gene expression. The ability to manipulate genes has led to rational, deliberate alterations of the genome of crop plants for improvement of their agronomic importance.

1.5.1 *Agrobacterium*-plant DNA transfer

The *Agrobacterium*-plant DNA transfer system is widely utilized for plant molecular and genetic engineering (Brown, 1990; Zambryski, 1992). This method depends on the susceptibility of the target crop to *Agrobacterium* as well as the availability of a regeneration procedure in which *Agrobacterium* transformation can be targeted to cells capable of regeneration (Draper *et al*., 1988; Hinchee *et al*., 1988).

The soil-born phytopathogen *Agrobacterium* (*A*). *tumefaciens* causes tumour diseases in most dicotyledonous and gymnospermous plants (De Cleene and De

Ley, 1976, de Framond *et al*., 1983; Hoekema *et al*., 1983; Draper *et al*., 1988; Prescott *et al*., 1990). The virulent strains of *A. tumefaciens* contain large tumour-inducing (Ti) plasmids which cause the disease "crown gall", and which interfere with the normal growth of an infected plant (Gasser and Fraley, 1989; Brown, 1990; Prescott *et al*., 1990; Kerr, 1992; Zambryski, 1992). These virulent strains infect plant wounds where they detect the presence of specific phenolic compounds of plant origin (Draper *et al*., 1988). The virulence (*vir*) genes present on the Ti plasmids of *A. tumefaciens* are then induced. Expression of the *vir* genes in the bacterium leads to the transfer of a discrete segment of the Ti plasmid DNA, called transferred-DNA (T-DNA), which is bounded by 25 bp repeat sequences, to plant cells where it is integrated into the host plant nuclear genome (Bevan, 1984; Brown, 1990; Gasser and Fraley, 1992) and transcribed into messenger RNA (de Framond *et al*., 1983; Draper *et al*., 1988). Expression of the integrated T-DNA oncogenic genes leads to the development of a tumour in the infected plant through an increase in growth hormone biosynthesis by the transformed cells (Zambryski *et al*., 1983; Gasser and Fraley, 1992). Expression of the T-DNA also elicits the production by these transformed cells of specific secondary metabolites, called opines.

This natural genetic transformation system has been exploited in the construction of a variety of highly efficient and sophisticated co-integrative and binary cloning vectors for transferring novel genes to plants (de Framond *et al*., 1983; Hoekema *et al*., 1983; Zambryski *et al*., 1983; Bevan, 1984; Klee *et al*., 1985; Brown *et al*., 1990). Early experiments in the 1980s demonstrated that no portion of a Ti-plasmid's T-DNA other than its borders are essential for DNA transfer into plant

cells and integration into the nuclear DNA (de Framond *et al*., 1983; Klee *et al*., 1985; Draper *et al*., 1988). This led to the adaptation of naturally occurring Ti plasmids to act as gene vectors for plant transformation (Draper *et al*., 1988). Efficient plant transformation vectors were developed by the removal of the T-DNA oncogenes and their replacement with antibiotic resistance genes (typically the neomycin phosphotransferase II (*npt II*) gene which confers kanamycin resistance), expression cassettes, reporter genes (typically β-glucuronidase) (Zambryski *et al*., 1983; Bevan, 1984; Fraley *et al*., 1985; Draper *et al*., 1988; Rogers *et al*., 1989; Gasser and Fraley, 1992) and more recently green fluorescent protein (*GFP*) which function in plants (Molinier *et al*., 2000; Murray *et al*., 2004; Bastar *et al*., 2004). Foreign DNA sequences introduced into plants using Ti plasmids have been shown to be stably inherited in Mendelian manner (Otten *et al*., 1981; Horsch *et al*., 1984).

Agrobacterium-mediated DNA transfer offers several advantages, compared to direct gene delivery methods. The advantages include defined integration of transgenes and preferential integration into transcriptionally active regions of the chromosome (Hiei *et al*., 2000). Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number of the transgene (Enriquez-Obregon *et al*., 1998; Hiei *et al*., 2000), potentially leading to fewer problems with transgene co-suppression and instability. The use of this transformation system normally results in clonally transformed plants. However, multiple insertions, rearrangements and/or deletions in the integrated genes have been detected in transformed tobacco (Scorza *et al*., 1994), cassava

(Schopke *et al*., 1996) and African violet (Mercuri *et al*., 2000) that had been transformed with *Agrobacterium* vectors.

Agrobacterium therefore constitutes an excellent system for introducing foreign genes into plants. To date cells of several plant species have been successfully transformed via the *Agrobacterium*-mediated gene transfer method (Zambryski *et al*., 1983; Horsch *et al*., 1984; Rogers *et al*., 1986; De Block *et al*., 1989; Raineri *et al*., 1990; Park *et al*., 1996; Clemente *et al*., 2000; Sarria *et al*., 2000; Cheng and Punja, 2002; Khan *et al*., 2003; Hoshi *et al*., 2004; Manickavasagam *et al*., 2004; Murray *et al*., 2004; Zeng *et al*., 2004; Jin *et al*., 2005; Sanjaya *et al*., 2005; Tohidfar *et al*., 2005; Travella *et al*., 2005).

Although *Agrobacterium*-mediated DNA transfer has proved to be efficient and is presently the method of choice for delivery of DNA into many types of plants, its host range is limited (Gasser and Fraley, 1989; Zambryski, 1992; Hiatt, 1993). Low efficiency of transformation, problems with the removal of *Agrobacterium* following transformation and manipulations of DNA in wide host range plasmids has been reported (Finer and McMullen, 1990). A large number of plant species, in particular the graminaceous monocotyledons are not natural hosts for *Agrobacterium* and cannot be routinely transformed with *Agrobacterium* based systems (Gasser and Fraley, 1989; Potrykus, 1991; Gasser and Fraley, 1992). More recently, however, efficient transformation of rice, maize, sorghum, cassava, canola, soybean and barley mediated by *Agrobacterium* was developed (Hiei *et al*., 1994; Ishida *et al*., 1996; Zhong *et al*., 1998; Sarria *et al*., 2000; Khan *et al*., 2003; Zeng *et al*., 2004; Travella *et al*., 2005).

There have been reports where frequency of transformation mediated by *A. tumefaciens* can be greatly enhanced by first generating microwounds in the target tissue through high-velocity microprojectile bombardment with uncoated particles (Bidney *et al*., 1992; Knittel *et al*., 1994; Brasileiro *et al*., 1996) or particles coated with *Agrobacterium* (Cordero de Mesa *et al*., 2000); cutting leaf tissue (Sunikumar *et al*., 1999); shaking with glass beads (Grayburn and Vick, 1995) or with carborundum (Cheng *et al*., 1996) and scratching with sandpaper (Hoshi *et al*., 2004). de Rhonde *et al*. (2001) reported a high transformation frequency of partially germinated soybean seeds subjected to vacuum infiltration in the presence of *A. tumefaciens*. Incubation with acetosyringone has also been reported to increase transformation frequencies (Godwin *et al*., 1991; Sunikumar *et al*., 1999; Sarria *et al*., 2000; Olhoft *et al*., 2001).

Agrobacterium is widely considered to be the only bacterial genus capable of transferring genes to plants. When suitably modified, *Agrobacterium* has become the most effective vector for gene transfer in plant biotechnology. However, the complexity of the patent landscape has created both real and perceived obstacles to the effective use of this technology for agricultural improvements by many public and private organizations worldwide. Many important crops, including species particularly relevant to the developing world, are also difficult to transform by *Agrobacterium*, difficult to regenerate from a susceptible explant, or prone to unsuitable genetic and phenotypic variation using current methodology (Broothaerts *et al*., 2005). Recently, Broothaerts *et al*. have shown that several species of bacteria outside the *Agrobacterium* genus can be modified to mediate gene transfer to a number of diverse plants. The plant-associated bacterial species

Rhizobium, *Sinorhizobium* and *Mesorhizobium*, have been made competent for gene transfer by acquisition of both a disarmed Ti plasmid and a suitable binary vector (Broothaerts *et al*., 2005). These bacteria interact with different cell types and stages, and as symbionts or benign endo- and epiphytes can be expected to evoke different plant responses. This alternative to *Agrobacterium*-mediated technology for crop improvement, in addition to affording a versatile 'open source' platform for biotechnology, may lead to new uses of natural bacteria-plant interactions to achieve plant transformation to previously intractable plant cell types, explants or species (Broothaerts *et al*., 2005).

1.5.2 Alternative methods for transferring genes to plants

Alternative methods for transferring genes to plants include direct gene transfer procedures (the most widely used next to *Agrobacterium* systems) (Schillito *et al*., 1985; Potrykus, 1991; Gasser and Fraley, 1992) and microinjection of DNA into cultured plant cells and intact plant organs (Gasser and Fraley, 1989; Uchimaya *et al*., 1989). The biolistic technique (microprojectile bombardment) has been most useful to date for the transfer of DNA into a wide variety of plant species for both transient gene expression and stable transformation studies (Cao *et al*., 1992; Finer *et al*., 1992; Hiatt, 1993; Rasmussen *et al*., 1994; Cabrera-Ponce *et al*., 1995; Zhou *et al*., 1995; Chen *et al*., 1998; Goldman *et al*., 2003; Srinivasa Reddy *et al*., 2003; Shu *et al*., 2005).

Microprojectiles can be used to transfer genes to a wide range of intact plant tissue (Hiatt, 1993) and this capability has permitted the genetic transformation of

several important crop species including soybean (McCabe *et al*., 1988; Finer and McMullen, 1991; Finer *et al*., 1992), cotton (Finer and McMullen, 1990; Klein *et al*., 1992), maize, wheat, rice (Klein *et al*., 1992, Chen *et al*., 1998; Zhang *et al*., 2002), papaya (Cabrera-Ponce *et al*., 1995), tobacco (Rasmussen *et al*., 1994), cassava (Zhang *et al*., 2000a), pearl millet (Goldman *et al*., 2003), perennial grass (Shu *et al*., 2005) and barley (Travella *et al*., 2005).

Another approach to plant transformation has been to incubate various plant parts (e.g. seeds, organs, tissues, cell cultures and pollen) in DNA solutions in the hope that the DNA will pass through the cell walls and integrate into the plant genome (Gasser and Fraley, 1989; Potrykus, 1991). Liposome-mediated gene delivery into plant cells is an established method for production of transgenic plants (Gad *et al*., 1990). Microlasers have been used to burn holes in the walls of pollen cells and tissues in an attempt to make openings for DNA entry (Weber *et al*., 1988). Another method of transferring DNA into plant cells is the use of viral vectors (Potrykus, 1991).

1.6 Transgene expression and gene silencing

The epigenetic phenomenon of transgene silencing is a significant observation for plant scientists and molecular biologists. The silencing of transgenes has received particular attention in plants, because it reduces the reliability of transgenic approaches for genetic improvement of crops (Srinivasa Reddy *et al*., 2003). With the rapidly increasing application of transgene technology in plants, the control of transgene expression has become an important point of concern.

Transgenic loci introduced into higher plant species frequently display unpredictable patterns of inheritance and expression. Transgene instabilities- either of inheritance or expression- complicate the identification, selection, and use of transgenic lines (Bregitzer and Tonks, 2003).

Many examples of gene silencing in transgenic plants have been described in recent years (Flavell, 1994; Matzke and Matzke, 1995; Repellin *et al*., 2001; Bregitzer and Tonks, 2003; Srinivasa Reddy *et al*., 2003; Travella *et al*., 2005). Some examples are due to post-transcriptional effects, transcriptional down regulation and DNA methylation. Plant tissue culture/regeneration protocols are known to cause genetic and epigenetic alterations in plants, including mutant phenotypes (Schmulders *et al*., 1995), chromosomal rearrangements (Peschke *et al*., 1987; Evans, 1989; Schmulders *et al*., 1995), activation of dormant transposons (Peschke *et al*., 1987), somaclonal variation and DNA methylation (Schmulders *et al*., 1995). Instability of gene expression could be related to the formation of a mosaic pattern within morphologically uniform tissues (Neuhuber *et al*., 1994; Bastar *et al*., 2004). Several reports have found that the incidence of gene silencing increases with transgene dosage (de Carvalho *et al*., 1992; Hart *et al*., 1992; Hobbs *et al*., 1993; Matzke and Matzke, 1995; Goodwin *et al*., 1996; Kumpatla and Hall, 1998). Transgene transcription also increases with increasing transgene dosage (de Carvalho *et al*., 1992; Goodwin *et al*., 1996). Transgene integration patterns and DNA rearrangements before and after insertion can be influenced by the structure of the transforming DNA. For example, it was recently shown that a 19 bp imperfect palindromic sequence of the CaMV 35S promoter constitutes a recombination hotspot (Kohli *et al*., 1999). Thus the

choice of promoter to drive transgene expression may have a large impact on the occurrence of silencing (McCabe *et al*., 1999). High activity from the promoter is correlated with hypermethylation and abolishment of tanscription as previously reported for dicotyledonous plants (Matzke and Matzke, 1995). Inheritance patterns of transgenes also exhibit irregular behaviour, and frequently deviate from Mendelian expectations (Bregitzer and Tonks, 2003).

1.7 Plant *in vitro* culture and plant regeneration

Plant regeneration is a critical step in the success of any crop improvement programme entailing *in vitro* culture. *In vitro* culture is also a way to study the mechanisms by which cells differentiate, thereby providing an experimental approach to link genotype with phenotype. As a consequence, there is an intense general interest in plant *in vitro* culture, which has been reflected in numerous symposia and reviews. Moreover, the discussion of the uses of *in vitro* culture for the future of crops has become a popular feature for many periodicals. Plant *in vitro* culture permeates plant biotechnology and cements together its various aspects, to a large extent the plant *in vitro* culture revolution has occurred because of the needs of this new plant biotechnology.

Plant regeneration is a morphogenic response to a stimulus that results in the production of organs, embryos and whole plants (Cassels, 1991). Plant regeneration can be achieved in two ways: through organogenesis or through somatic embryogenesis. Organogenesis is a process of differentiation by which plant organs are formed *de novo* or from pre-existing structures, while

embryogenesis is the process of embryo initiation and development (Shoemaker *et al*., 1986).

Many explants of diverse origins from plants belonging to different taxonomic groups have been established in culture and the factors controlling growth and differentiation in several tissues ascertained (Saeed, *et al*., 1997; Reichert *et al*., 2003; Rauf and Rahman, 2005; Rodrigues *et al*., 2005). The subtle interactions among different groups of plant hormones, auxins, cytokinins, gibberellins, abscisin and ethylene operate either directly or indirectly, alone or in synergistic combinations have given an insight into the interrelationships existing between cells, tissues and organs, and in the integrated development of a whole plant (Cassels, 1991).

Studies of specific species done by various researchers (Cheng *et al*., 1980; van der Hoeven *et al*., 1994; Rauf and Rahman, 2005) have shown that several factors, namely medium composition, hormonal content, source of explant, physiological age of the explant and environmental conditions influence the growth of *in vitro* cultures and the recovery of fertile plants. Within a species, genotype is probably the most critical factor for obtaining successful plant regeneration (Dhir *et al*., 1992; Rauf and Rahman, 2005).

For legumes that have been regarded as recalcitrant to transformation, regeneration *in vitro* is highly genotype specific and only rarely are cultivated varieties amenable to regeneration. In these cases, regeneration remains more of an 'art' than a science (Grant *et al*., 2003). In addition, regeneration is often slow

and the frequency of transformation (number of transformed plants generated from each explant) is often low (Somers *et al*., 2003). Expanding the range of genotypes within a species that undergo the requisite tissue culture process would provide a major contribution to improving the transformation system (Somers *et al*., 2003).

The shoot apical meristem presents an excellent tissue for plant regeneration, particularly in species for which a tissue culture regeneration is not well established and thus possess considerable potential for genotype independent gene transfer to recalcitrant species (Potrykus, 1990). All the species for which micropropagation protocols have been developed, the greatest number has been on axillary bud proliferation (Draper *et al*., 1988).

The shoot apical meristem of plants is the distal-most portion of the shoot apex and is typically domed -shaped group of 800-1200 cells approximately 100 μm in diameter. However, the apical meristem size varies with species and age (Medford *et al*., 1991). The shoot apical meristem in most dicotyledons contains two major cell types, the tunica layer (usually two layers thick, LI and LII) and the corpus (LIII). Cell division in the tunica layer layers is mainly in the anticlinal plane, whereas, cell division in the corpus is both anticlinal and periclinal. Therefore, the shoot apical meristem maintains discrete cell layers. The LI cell layer gives rise to the epidermis of the plant while the LII layer gives rise to the palisade parenchyma and the spongy parenchyma. Moreover, the LII is the cell layer that is responsible for the formation of male and female gametes. The LIII (or corpus) layer gives rise to the central stem tissue such as the pith

(Marcotrigiano, 1990). The individual cells are dividing, expanding and differentiating (Grandjean *et al*., 2004). The shoot meristems continuously provide new cells for developing shoot organs. Because the meristematic dome functions to proliferate cells as well as to initiate new tissues and organs, genes expressed in meristems may also be expressed in other tissues (Medford *et al*., 1991).

Efficient and reproducible plant regeneration systems have been developed from shoot apical meristems of aseptically germinated seedlings of sorghum (Zhong *et al*., 1998), castor (Sujatha and Reddy, 1998), pearl millet (Devi *et al*., 2000), soybean (Kartha *et al*., 1991; Christou *et al*., 1989; Sato *et al*., 1993), cotton (Gould *et al*., 1991a; McCabe and Martinell, 1993; Saeed *et al*., 1997; Hemphill *et al*., 1998; Zapata *et al*. 1999 a and b) and pear (Matsuda *et al*. 2005).

Various researchers have reported transformation of shoot apical meristems and stable integration, expression and inheritance of the transgene. Plant species which have been transformed by *A. tumefaciens* using shoot apical meristems as explants include: *Zea mays* (corn) (Gould *et al*., 1991b), rice (Park *et al*., 1996), lupin (Li *et al*., 2000), pear (Matsuda *et al*., 2005) and cotton (Zapata *et al*., 1999a; Satyavathi *et al*., 2002; Satyavathi *et al*., 2005). Satyavathi *et al*. (2002) produced transgenic cotton plants that were not mosaics.

Shoot apical meristems of soybean (Christou *et al*., 1989; Sato *et al*., 1993), oat and barley (Zhang *et al*., 1999b) have been transformed using electric discharge particle acceleration. More recently, transformation of shoot meristematic

cultures by particle bombardment (Zhang *et al*., 2002; Cho *et al*., 2003) and *Agrobacterium*-mediated DNA transfer (Saini and Jaiwal, 2005) have been reported.

1.8 Regeneration and transformation of soybean

Soybean (*Glycine max* L. Merrill) is native to Eastern Asia and regarded as one of the five sacred grains of ancient China (Barwale and Widholm, 1990). Today, it is an extremely important international annual grain legume crop (Lippmann and Lippmann, 1984; Chee *et al*., 1989; Christou, 1997; Somers *et al*., 2003). Soybean's introduction to the West occurred many centuries after its initial domestication in Northern China in the 11th Century BC (Probst and Judd, 1976). Having evolved in the Far East, there were no varieties well suited to Western agricultural environments, and early interest in cultivating this legume as a grain crop soon flagged. As more was learned about this plant, its real value became increasingly apparent. Breeders in Western countries were encouraged to intensify their research, which led to the production of varieties better adapted to their particular areas. Soybean has a wide adaptability to many growing regions (Somers *et al*., 2003). Today, soybean is a world crop, cultivated widely in the U.S.A., Brazil, Argentina, China, India and South Africa. Transformation is emerging as an important crop improvement tool for soybean (Somers *et al*., 2003). Roundup Ready soybean cultivars have captured a major stake in market share of soybeans planted in the US and Argentina (Somers *et al*., 2003).

The soybean, rich in seed protein (range 30-48%, average 40%) and oil (range 13-22%, average 20%) (Singh and Hymowitz, 1999) is an economically important leguminous seed crop for high protein food and vegetable oil for human consumption, and of protein-rich livestock feed (Chee *et al*., 1989; Rech *et al*., 1989; Widholm *et al*., 1992; Singh and Hymowitz, 1999; Zeng *et al*., 2004). Soybean is ranked number one in the world oil production (48%) in the international trade markets among the major crops, namely, cottonseed, peanut (groundnut), sunflower seed, rapeseed, coconut and palm kernel (Singh and Hymowitz, 1999). The demand for soybean, being the most produced, traded and utilized crop in the world, has resulted in increased soybean production and research on this crop has also increased proportionally (Barwale and Widholm, 1990; Somers *et al*., 2003).

Soybean is cultivated successfully in many parts of South Africa, and the importance of this crop is illustrated by the fact that the area planted with this legume increases annually (http://www.nda.agric.za). In addition to animal feed requirements, demands for a source of cheap protein by our expanding population have resulted in a sharp increase in South African soybean production over the past few years. Soybean competes favourably with maize on an economic basis (Duxbury *et al*., 1990) and there is considerable potential for expanding soybean production in South Africa.

Soybean has been a prominent target for improvement by conventional plant breeding methods (Hinchee *et al*., 1988; McCabe *et al*., 1988; Chee *et al*., 1989). However, the limited genetic base in domestic soybean cultivars has restricted the

power of traditional breeding methods to develop varieties with improved or value-added traits (Hinchee *et al*., 1988; Singh and Hymowitz, 1999). It is for this reason that *in vitro* soybean cultures have been developed and molecular techniques used to incorporate or modify genetic traits that cannot be introduced or modified by classical plant-breeding methods. At present, genetic manipulation of soybean through recombinant technology is severely limited by the lack of an efficiently coupled transformation and regeneration system for this species. It would therefore be beneficial to establish a working system for genetically engineering South African grown varieties as this would facilitate the production of cultivars with improved characteristics such as resistance to environmental stress and disease, enhanced nutritional value, as well as tolerance to specific herbicides.

Many attempts to develop *in vitro* regeneration protocols have been reported, but despite these efforts, soybean and related species of this genus have been amongst the more difficult plant species from which to achieve regeneration of cells and plants in tissue cultures (Widholm *et al*., 1992). The major impediments to the development of a regeneration system for soybean have been genotype and the low rate of regeneration from explants. It has been reported that grain legumes have been, in general, a lot more recalcitrant to *in vitro* manipulation than other species (Christou, 1997).

Plant regeneration from somatic embryogenesis as a principal mode of *in vitro* regeneration for soybean was described by Christianson *et al*. (1983) where plants were regenerated from a soybean suspension culture through embryogenesis at a

low frequency from an immature embryo-derived culture. Lippmann and Lippmann (1984) induced somatic embryogenesis from cotyledons of immature embryos which did not develop further. Other more successful embryogenic regeneration systems have been described by Lazzeri *et al*. (1985), Ranch *et al*. (1985), Barwale *et al*. (1986) and Lippmann and Lippmann (1993), Santarem and Finer (1999), Aragao *et al*. (2000), Yan *et al*. (2000) and Walker and Parrot (2001). These researchers were able to produce somatic embryos which were able to develop into whole plants from different genotypes using callus cultures derived from immature embryos. Ghazi *et al*. (1986) induced both a smooth and a rough callus from which embryogenic cultures were produced from the smooth type and whole plantlets were obtained. An embryogenic, regenerable suspension culture has also been described by Finer (1988), Finer and Nagasawa (1988) and Sato *et al*. (1993). More recently Sairam *et al*. (2003) reported on the regeneration of soybean plants from cotyledonary nodal callus.

Soybean plant regeneration has also been possible through organogenesis, using epicotyls (Wright *et al*., 1987b) and shoot apical meristems (Kartha *et al*., 1981; Barwale *et al*., 1986, McCabe *et al*., 1988; Sato *et al*., 1993). Plant regeneration from cotyledonary nodes (Cheng *et al*., 1980; Wright *et al*., 1986; Kaneda *et al*., 1997; Zhang *et al*., 1999a; Olhoft and Somers, 2001; Shan *et al*., 2005), hypocotyls (Kaneda *et al*., 1997; Dan and Reichert, 1998; Reichert *et al*., 2003), cotyledons (Hinchee *et al*., 1988) and primary leaf tissue (Wright *et al*., 1987a) has also resulted in reliable methods of regeneration of fertile soybean plants. Multiple shoots have been obtained from genotypes tested and resulted in the generation of complete plants (Kaneda *et al*., 1997). In the study done by

Reichert *et al*. (2003) no morphological differences were noted among regenerants, or between them and seed initiated plants. All regenerants produced viable seed that germinated and produced morphological normal plants (Reichert *et al*., 2003). Several groups have reported plant regeneration from protoplasts isolated from various tissues, namely roots, leaves (Wei and Xu, 1988), immature embryos and cotyledons (Myers *et al*., 1989; Dhir *et al*., 1992; Widholm *et al*., 1992) of both wild and cultivated soybean. More recently, Rodrigues *et al*. (2005) reported on the embryogenic responses in soybean anther culture.

Foreign DNA has been incorporated into the soybean genome resulting in transformed plants (Shan *et al*., 2005). One of the major impediments to the development of a transformation system for soybean has been the low (0.4-3%) efficiency of introducing foreign DNA into soybean cells (Hinchee *et al*., 1988; Sato *et al*., 1993; Donaldson and Simmonds, 2000). Soybean plant transformation has been accomplished using *Agrobacterium*-mediated transfer systems (Hinchee *et al*., 1988; Parrot *et al*., 1989; Di *et al*., 1996; Zhang *et al*., 1999a; Clemente *et al*., 2000; Yan *et al*., 2000; Ke *et al*., 2001; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Ko *et al*., 2004; Zeng *et al*., 2004; Shan *et al*., 2005), direct infection of germinating seeds with *Agrobacterium* (Chee *et al*., 1989; de Rhonde *et al*., 2001) and with DNA-coated microprojectiles (McCabe *et al*., 1988; Christou *et al*., 1989; Finer *et al*., 1992; Sato *et al*., 1993; Moore *et al*., 1994; Parrot *et al*., 1994; Hadi *et al*., 1996; Stewart *et al*., 1996; Ponappa *et al*., 1999; Santarem and Finer, 1999; Aragao *et al*., 2000; Srinivasa Reddy *et al*., 2003). Transgenic plants have been obtained with each technique and the foreign genes inserted into these plants were stably inherited in the progeny of the transformed plants (McCabe *et al*.,

1988; Clemente *et al*., 2000; Yan *et al*., 2000; Olhoft *et al*., 2003; Zeng *et al*., 2004).

Soybean protoplasts have been stably transformed using electroporation (Christou *et al*., 1987; Dhir *et al*., 1991; Dhir *et al*., 1992; Widholm *et al*., 1992; Christou 1997) and co-cultivation with *Agrobacterium* (Baldes *et al*., 1987), but plants could not be regenerated from the callus in these cases. Molecular and biological analysis of protoplast-derived non-transgenic and transgenic soybean plants suggests that protoplast culture could be a valuable tool in soybean improvement (Dhir *et al*., 1992).

The *Agrobacterium*-mediated transfer system affords the most efficient recovery of transformed soybean plants (Wright *et al*., 1987b). While gall formation on soybean has been documented (Pedersen *et al*., 1983; Byrne *et al*., 1987; McKenzie and Cress, 1992; Bailey *et al*., 1994; Bond *et al*., 1996) and as discussed above generation of transgenic soybean through *Agrobacterium*-mediated gene transfer has been accomplished, the efficiency is low and soybean wound tissue remains susceptible to *Agrobacterium* infection for a short period of time (Kudirka *et al*., 1986). Further, susceptibility to *Agrobacterium* is highly genotype specific (Owens and Cress, 1985; Bond *et al*., 1997), limiting the ability to introduce novel genes directly into elite cultivars. Soybean cultivars have been transformed successfully using either cotyledonary node (Di *et al*., 1996; Clemente *et al*., 2000; Donaldson and Simmonds, 2000; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Zeng *et al*., 2004; Shan *et al*., 2005) or somatic embryogenesis

(Ko *et al*., 2004) based transformation methods, the two predominant soybean transformation methods.

Efforts to overcome problems with host/tissue specificity of *Agrobacterium* and low transformation efficiency include use of hypervirluent/modified *Agrobacterium* strains, adding thiol compounds to the co-cultivation medium, or sonicating target tissues prior to co-cultivation (Hood *et al*., 1993; Trick and Finer, 1997; Trick and Finer, 1998; Ke *et al*., 2001; Olhoft and Somers, 2001).

Soybean transformation methods are not routinely reproducible (Christou, 1997). Soybean transformants are often sterile and sterility is attributed mostly to chromosomal abberations (Singh and Hymowitz, 1999). Frequently, unexpected segregations and low expression or disappearance of foreign genes have been observed (Singh and Hymowitz, 1999). Genes may be physically present but gene activity may be poorly expressed or totally lost in subsequent generations. This is generally attributed to co-suppression or gene silencing. Srinivasa Reddy *et al*. (2003) reported on gene silencing in transgenic soybean plants transformed via particle bombardment containing multiple copies of the transgene.

Broadening the gene pool of soybean via plant gene transfer techniques has been accomplished for such traits such as herbicide tolerance (Padgette *et al*., 1995; Zhang *et al*., 1999a; Clemente *et al*., 2000; Olhoft and Somers, 2001), amino acid modification (Falco *et al*., 1995), insect resistance (Parrot *et al*., 1994; Stewart *et al*., 1996) and viral resistance (Di *et al*., 1996).

Routine recovery of transgenic soybean plants has been restricted to a few genotypes (Di *et al*., 1996; Clemente *et al*., 2000; Donaldson and Simmonds, 2000; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Zeng *et al*., 2004; Shan *et al*., 2005) with no reports of transformed South African grown Ibis or Talana cultivars. Therefore, development of an efficient regeneration and transformation protocol for these cultivars will greatly aid soybean transgenic technology in general.

1.9 Regeneration and transformation of cotton

Cotton (*Gossypium hirsutum* L.) is a major fibre crop worldwide and its economic importance is increasing annually (Regier *et al*., 1986; Perlak *et al*., 1990; John, 1997; Ganesan and Jayabalan, 2004). Cotton is of particular importance in the textile industry, with nearly 50 % of the total textile fibre consumption depending on cotton fibres (Peeters *et al*., 1994). Cotton is also cultivated for its essential seed oil (Ganesan and Jayabalan, 2004), the world's second most important oilseed crop after soybean (Nobre *et al*., 2001). In addition to textile manufacturing, cotton and cotton by-products provide raw materials that are used to produce a wealth of consumer-based products, foodstuffs, livestock feed, fertilizer and paper (Mishra *et al*., 2003). The production, marketing, consumption and trade of cotton-based products further stimulates the economy, and based on the revenues in excess of $100 billion generated annually in the U.S. alone, cotton is the number one value-added crop (Mishra *et al*., 2003).

Cotton is grown on 2.5 % of the arable land in five continents. It is cultivated and grown mainly under subtropical and tropical environmental conditions (Nobre *et al*., 2001). It is grown in more than 80 countries and has an annual production of 20 million tons (Li *et al*., 2004). Among the cotton producing countries, India ranks first in production and area in cultivation, providing 32% of the world's total area of cotton cultivation, followed by the U.S.A. (23%) and China (20%) (Benedict and Altman, 2001). It has been estimated that 180 million people depend, either directly or indirectly, on the production of cotton for their livelihood (Benedict and Altman, 2001). Cotton production is of high economic importance in Africa. Cotton is grown on 2.5 million ha of the continent, most of it on small plots of less than 10 ha (Scott, 2004). In 2004-2005 in South Africa, cotton was grown on 36 303 ha of land in various regions by both commercial and small-scale farmers (http://www.cottonsa.org.za). Cotton production areas in South Africa are the Northern Cape-Lower Orange River and Griqualand West, North West-Rustenberg, Northern Province- Limpopo Valley, Loskop and Springbok Flats, Mpumalanga and KwaZulu-Natal (http://www.cottonsa.org.za).

A rich genetic reservoir is available in wild and wild relatives of cotton, but genetic improvement through inter-specific hybridization is hampered by incompatibility barriers. There has long been interest in the genetic improvement of this valuable crop species (John, 1997). Cotton species are continually being improved by traditional (conventional) breeding techniques and a number of programmes have been developed aimed at improving existing genotypes using genetic engineering techniques (Llewellyn *et al*., 1990; Nobre *et al*., 2001; Mishra *et al*., 2003). Cotton biotechnology plays a vital role in improving the quality as

well as the quantity of fibre by producing plants resistant to insect, herbicide, fungi, bacteria and nematodes through genetic engineering (Pannetier *et al*., 1997; Leelavathi *et al*., 2004).

Genetically modified cotton is commercially grown in the U.S.A (May *et al*., 2004). Growers have overwhelmingly adopted glyphosate-resistant (Roundup Ready, Monsanto Co., Chesterfield, MO) cotton cultivars since introduction in 1997 (May *et al*., 2004). In 2002, about 72% of the U.S.A. hectarage was planted to cultivars containing the Monsanto glyphosate gene alone, or combined with Monsanto's Bollgard gene (derived from *Bacillus thuringiensis* var. *kurstaki*) conferring protection from certain lepidopteran pest insects (Perlak *et al*., 1991; USDA-AMS, 2002). The popularity of glyphosate-resistant cotton cultivars reflects the broad spectrum weed control possible with glyphosate, plus capability to farm cotton on more hectares compared with traditional weed management approaches (Culpepper and York, 1998; York, 1997). Liberty Link cotton event 25 (PPT-resistant cotton) has been commercialized in the U.S.A by Bayer Crop Science (http://www.ogtr.gov.au). The genetically-modified cotton has only one introduced gene, the herbicide tolerance gene (*bar*). The *bar* gene expresses a protein that provides tolerance to glufosinate ammonium, the active ingredient in the herbicide Liberty, and enables the herbicide to be applied for weed control in the genetically modified crop (http://www.ogtr.gov.au).

Regeneration *in vitro* of *Gossypium hirsutum* has been a much sought after goal that would make feasible the use of somaclonal variation and gene manipulation for improvement of existing breeding lines (Trolinder and Goodin, 1988a). The

regeneration of cotton plants from tissue culture has however been notoriously difficult and genotype has been shown to play an important role in cotton regeneration (Gawel *et al*., 1986; Shoemaker *et al*., 1986; Trolinder and Xhixian, 1989; Rauf and Rahman, 2005). Efficient *in vitro* techniques for the regeneration of large numbers of plantlets from cotton are limited when compared to other major commercial crops (Kumria *et al*., 2003).

Embryogenesis and subsequent plant regeneration has been obtained in *G. hirsutum* from callus produced from immature tissues of immature organs, namely cotyledons (Davidonis and Hamilton, 1983; Firoozabady *et al*., 1987; Ganesan and Jayabalan, 2004), immature embryos (Rangan *et al*., 1984), immature embryos and hypocotyls (Mitten, 1985; Shoemaker *et al*., 1986), seedling hypocotyls (Gawel *et al*., 1986; Pannetier *et al*., 1997; Kumria *et al*., 2003; Ganesan and Jayabalan, 2004; Tohidfar *et al*., 2005) and cotyledonary leaf sections (Kumria *et al*., 2003). More recently, researchers have reported somatic embryogenesis and plant regeneration in several cotton cell lines (Rauf and Rahman, 2005; Sakhanokho *et al*., 2005). Somatic embryogenesis from mature tissues (leaf, petiole and stem) has also been reported (Gawel *et al*., 1986; Trolinder and Goodin, 1988b; Zhang *et al*., 2000b). Nobre *et al*. (2001) reported on the regeneration of embryogenic callus and plantlets from stomatal guard cells.

Regeneration through somatic embryogenesis is preferred over organogenesis because of the probable single-cell origin of somatic embryos (Merkle *et al*., 1995), thus reducing the chimeric transformation events. Although plant regeneration efficiency via somatic embryogenesis has been improved to 75.8%

(Ganesan and Jayabalan, 2004), genotype-dependent response, a prolonged culture period, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, and a lack of shoot elongation are the problems associated with cotton regeneration (Kumria *et al*., 2003). Other several problems have been highlighted during the regeneration of cotton from somatic embryos, including the secretion of secondary metabolites from the explants in the medium, browning of callus after a short period of culture, a low frequency of embryo maturation and abnormal somatic embryo germination (Ganesan and Jayabalan, 2004). The majority of the reports on *in vitro* regeneration of cotton via somatic embryogenesis pertain to either wild or Coker varieties of *G. hirsutum* that are not highly cultivated (Agrawal *et al*., 1997).

Earlier attempts to culture cotton protoplasts isolated from cotyledons or hypocotyl callus resulted in the formation of microcolonies or callus, but no plants were regenerated (Firoozabady and DeBoer, 1986; Saka *et al*., 1987). However, Peeters *et al*. (1994) has reported plant regeneration from cell suspension-derived protoplasts of *G. hirsutum* using feeder cells. The protoplast-to-plant development system reported (Peeters *et al*., 1994) might become an important component of cotton improvement through techniques such as gene transfer, organ transfer and somatic hybridization. Additional research is needed however to verify whether this method is applicable to other cotton species and to evaluate the true-to-type nature of the regenerants. More recently, Sun *et al*. (2005) reported that fertile regenerated plants were obtained from protoplasts via somatic embryogenesis.

Organogenesis and subsequent plant regeneration from the shoot apical meristems of seedlings (Gould *et al*., 1991a; McCabe and Martinell, 1993; Saeed *et al*., 1997; Hemphill *et al*., 1998; Zapata *et al*., 1999a and b; Satyavathi *et al*., 2002), cotyledonary nodes (devoid of cotyledons and apical meristems) (Agrawal *et al*., 1997), cotyledonary nodes (including the shoot apex) (Gupta *et al*., 1997), hypocotyl explants (Mishra *et al*., 2003) and embryonic axes (Morre *et al*., 1998) have been reported. Shoot regeneration from the shoot apical meristem is direct, relatively simple and is not prone to somaclonal variation and chromosomal abnormalities (Gould *et al*., 1991a). It has also been reported that root organogenesis in cotton shoots is genotype dependent and highly variable (10-93%) (Gould *et al*., 1991a; Hempill *et al*., 1998) and this presents a significant bottleneck in the overall recovery of plants from culture.

Regeneration of somatic embryos, used together with the callus-based transformation procedure developed by Horsch and collegues (1985), has been successfully used in *Agrobacterium*-mediated transformation of cotton (Firoozabady *et al*., 1987; Umbeck *et al*., 1987; Perlak *et al*., 1990; Bayley *et al*., 1992; Pannetier *et al*., 1997; Sachs *et al*., 1998; Kohel and Yu, 1999; Leelavathi *et al*., 2004; Jin *et al*., 2005; Tohidfar *et al*., 2005) and in particle bombardment procedures (Finer and McMullen, 1990; Rajasekaran *et al*., 1996; Rajasekaran *et al*., 2000) to obtain insect, herbicide and disease resistant plants. Fertile, phenotypically normal transformed plants have been produced by these methods of transformation and foreign genes in cotton are inherited and expressed in a Mendelian fashion (Tohidfar *et al*., 2005). However, these advances presently remain restricted to the cultivars that can be regenerated from calli and cell

suspensions by somatic embryogenesis. Mostly, only Coker varieties have been transformed and regenerated consistently (Trolinder and Goodin, 1987; Firoozabady *et al*., 1987; Umbeck *et al*., 1987). Published *Agrobacterium*-mediated methods require approximately 10-12 months, or longer, to regenerate transgenic cotton plants (Firoozabady *et al*., 1987; Umbeck *et al*., 1987; Trolinder and Goodin. 1987, 1988b). More recently, however, a method of transforming embryogenic callus with *Agrobacterium* only took 3-5 months to produce 67 transgenic cotton plants from an average of 75 globular embryo clusters (Leelavathi *et al*., 2004). There have also been reports on transformation of cotton by particle bombardment of embryogenic cell suspension cultures (Rajasekaran *et al*., 2000). High frequency (4%) stable transformation of cotton by particle bombardment of embryogenic cell suspension cultures has been obtained (Rajasekaran *et al*., 2000). However, these transgenic plants from cell cultures older than 6 months produced plants with abnormal morphology and a high degree of sterility.

Zapata *et al*. (1999a) and Satyavathi *et al*. (2002 and 2005) have reported on the transformation of cotton using *Agrobacterium* and the shoot apex. Transformation of elite cotton cultivars via particle bombardment of organized shoot tip meristems has been reported (Finer and McMullen, 1990; McCabe and Martinell, 1993; Chlan *et al*., 1995; Keller *et al*., 1997). Particle bombardment transformation protocols using the shoot apical meristem, although genotype-independent, are extremely laborious and the transformation frequencies for cotton by particle bombardment is low. Only 0.1 to 0.2% of bombarded meristems are recovered as transgenic plants (John, 1997) and the occurrence of

chimeric plants within the regenerates is common (McCabe and Martinell, 1993). A large number of meristems have to be transformed and, if the cultivar is not amenable to induction of multiple shoots and axillary branching, the transformed status of the plants can be analyzed only in the next generation (John, 1997). Recently, Majeed *et al*. (2000) reported on the transformation of shoot tip cultures using a combination of *Agrobacterium* and particle bombardment to obtain virus resistant transgenic cotton plants.

More recently, Li *et al*. (2004) have reported genetic transformation of cotton pollen by means of vacuum infiltration and *Agrobacterium*-mediated T-DNA transfer.

Somaclonal effects due to mutations during tissue culture and regeneration processes and/or the site of gene insertion into the plant genome have been found to influence gene expression in regenerated cotton lines (Sachs *et al*., 1998).

The production of transgenic cotton plants, using either *Agrobacterium* or particle gun bombardment, has been restricted to a few genotypes (Finer and McMullen, 1990; McCabe and Martinell, 1993; Chlan *et al*., 1995; Keller *et al*., 1997; Zapata *et al*., 1999a; Satyavathi *et al*., 2002) with no reports of transformed South African grown Sabie, LRCC 101, 107/1 or Palala cultivars. To aid South African cotton transgenic technology, development of an efficient regeneration and transformation protocol for these cultivars is necessary.

1.10 Aims of the research

The globe's natural resources are being placed under increasing pressure as demands for food and textiles increase. Increasing the productivity of crop species is dependent on many factors, including environmental conditions and the use of herbicides to control growth of weeds. These factors could adversely affect plant production. An enormous amount of research has been and continues to be devoted to *in vitro* methods for transferring genes into plants and regeneration using tissue culture. Several techniques have been devised, but no single method is applicable to all plant species, therefore it is very important to develop transgenic South African soybean and cotton cultivars suitable for different geographical regions. However, regeneration and transformation of these crops is difficult.

Due to growing economic potential of cotton and soybean, for both small-scale and commercial farmers in South Africa, the general objective of this research was to develop regeneration and transformation protocols for herbicide resistance for important cotton and soybean cultivars in South Africa.

Specific aims of the research:

1. a. A preliminary study to transform tobacco with the *pat* gene and then assess herbicide resistance in transformed tobacco plants and T1, T2 and T3 progeny.
   b. To assess the stability of the *pat* gene in transgenic tobacco plants through six cycles of propagation, high temperature regimes and drought stress.

This work done on tobacco, as a model crop, was to set the platform for further genetic manipulations with the more difficult crops, cotton and soybean.

2. a. To evaluate commercially grown soybean (Talana and Ibis) and cotton (107/1, Palala, LRCC 101 and Sabie) cultivars in South Africa for their regeneration potential *in vitro*.

   b. To engineer soybean and cotton genetically with the *pat* gene to attempt to establish resistance to the herbicide Basta (active ingredient, PPT).

   c. To confirm the presence of the inserted *pat* gene in transformed soybean and cotton plants.

C H A P T E R 2

Transformation of tobacco and the effect of six cycles of propagation and environmental stress on the stable expression of the phosphinothricin acetyltransferase (*pat*) gene in transgenic tobacco and T1,T2 and T3 plants.

Sections of this chapter have been presented at the following conferences:

1. BIOY2K Combined Millennium Meeting-Biotech 2000, Grahamstown, 23-28 January 2000.
   C. McNaughton and M.E.C. Rey. The effect of subculturing and environmental stress on the stable expression of the phosphinothricin acetyltransferase (*pat*) gene in transgenic tobacco plants.

2. Biotechnology for Africa 95-All African Conference on Biotechnology, University of Pretoria, South Africa, 13-15 November 1995.
   C.R. Viljoen, C. McNaughton and V.H. Whitlock. Genetic manipulations and transfer of the phosphinothricin acetyltransferase (*pat*) gene into tobacco and soybean

Abstract

The stability of transgene expression in transgenic plants is important in the biotechnology industry. Tobacco (*Nicotiana tabacum* cv. Samsun) leaf disks were transformed with *Agrobacterium tumefaciens* LBA4404 harbouring the recombinant vector pBI101 containing the *pat* gene and *npt II* marker gene. Transformed leaf disks were placed on MSD4X2 medium containing 250 µg/ml cefotaxime and 100 µg/ml kanamycin to select for growth of transformed plant cells and shoots. Non-transformed leaf disks did not produce shoots or callus and within 21 days showed signs of chlorosis and became necrotic. All putative transformed shoots subsequently rooted 100% in MS medium containing 250 µg/ml cefotaxime, 100 µg/ml kanamycin and either 0.5, 1, or 20 mg/l PPT, indicating that the *pat* gene was present in the transformed tobacco plants and successfully detoxifying the herbicide PPT. One hundred percent transgenic tobacco plants placed in potting mixture and acclimatized in the growth chamber were phenotypically normal, flowered and produced seed. PCR analysis confirmed the integration of the *pat* gene in all nine transgenic tobacco plants genome and the T1, T2 and T3 progeny. Transgenic tobacco parent plants (T0) and T1 transformed progeny sprayed with a 2% aqueous solution of the commercial herbicide Basta remained green and 100% were tolerant to the herbicide after two weeks, compared with the control plants which showed necrotic lesions within 48 hours. Three *in vitro* grown transgenic tobacco lines carrying the *pat* gene were propagated and maintained for 14 months *in vitro* through six cycles of propagation onto medium containing 50 µg/ml kanamycin

and 20 mg/l PPT using axillary buds to determine the stability of the *pat* gene *in vitro*. Within one week on this medium, axillary shoots developed which then rooted in medium containing 50 µg/ml kanamycin and 20 mg/l PPT, indicating the presence of the *pat* gene which was successfully detoxifying the herbicide PPT. The stability of the *pat* gene in the genome of the transgenic plants after each cycle of propagation was confirmed by the generation of the 558 bp *pat* coding region by PCR analysis. Transgenic tobacco plants grown in the growth chamber were subjected to temperatures of 30°C, 35°C, 40°C and drought stress, to determine the effect on *pat* gene expression. Leaves from tobacco plants were harvested after each temperature and drought stress experiment and the *pat* protein (PAT) detected and quantified using a PAT-ELISA kit. Drought-stressed tobacco plants were allowed to recover at the temperatures tested and then the PAT-ELISA was performed again to assess expression of the *pat* gene. All transgenic leaf samples produced a blue colour indicating that all the plants were producing PAT. The amount of PAT produced from non-stressed plants at 30°C, 35°C, 40°C was 7.7, 7.6 and 8.0 ng PAT/ml, respectively. The transgenic plants which were drought stressed at 30°C, 35°C and 40°C produced 7.5, 7.7 and 8.0 ng PAT/ml, respectively. The control plants at 25°C produced 7.5 ng PAT/ml. When the drought stressed plants were allowed to recover, the amount of PAT produced by the recovered plants did not differ from when they were stressed (7.6, 7.6 and 8.0 ng PAT/ml for temperatures 30°C, 35°C and 40°C, respectively). High temperatures and drought stress, therefore, had no adverse effect on the expression of the *pat* gene in the transgenic tobacco plants. The presence of the *pat* gene in these transgenic plants after the high temperature and drought stress experiments

was confirmed by regeneration of tobacco plantlets on medium containing 100 µg/ml kanamycin and 20 mg/l PPT and PCR analysis.

2.1 Introduction

Nicotiana (*N.*) *tabacum* has a high level of responsiveness in tissue culture and so has been the species of choice in many transformation experiments. Extensive series of data has been produced on the genetic analysis of transformants produced either by the use of *Agrobacterium* (*A.*) *tumefaciens* (Otten *et al*., 1981; De Block *et al*., 1987; Vyskot *et al*., 1989; Caligari *et al*., 1993; Mannerlof and Tenning, 1997; Kohne *et al*., 1998; Sunilkumar *et al*., 1999; Bastar *et al*., 2004; Tsai *et al*., 2005) or by direct gene transfer techniques (Potrykus *et al*., 1985). Several of these studies show that non-Mendelian as well as Mendelian segregation may occur amongst the progeny of the initial transformants and that in addition, the level of expression of the selectable marker gene may vary considerably amongst different transformants.

After *Agrobacterium*-mediated transformation, the T-DNA is randomly integrated into the plant genome and the relative location of the T-DNA can result in the fluctuation of the expression of the transgene (Mittelstein *et al*., 1991). Insertion of multiple T-DNA copies during the *Agrobacterium* transformation is a common phenomenon (Delores and Gardner, 1988). Expression of some or all of the copies can influence the level of expression of the transgene. Multiple T-DNA copies can be silenced either in the hemizygous or homozygous stage (de

Carvalho *et al*., 1992). Transgene expression can also be influenced by the relative location of the integrated T-DNA into the genome (called position effects) (Matzke and Matzke, 1993, Curtis *et al*., 2000); the chromatin structure; methylation state (Matzke and Matzke, 1995; Goodwin *et al*., 1996; Curtis *et al*., 2000); post-transcriptional regulation (Meyer, 1995); copy numbers (Curtis *et al*., 2000); truncations and rearrangements (Delores and Gardner, 1988). Instability of gene expression could be related to the formation of a mosaic pattern within morphologically uniform tissues (Neuhuber *et al*., 1994; Bastar *et al*., 2004). When compared with microprojectile bombardment, *Agrobacterium*-mediated transformation offers several advantages that help reduce unwanted gene silencing (Kohli *et al*., 1999). These include defined transgene integration, low transgene copy numbers and fewer rearrangements within inserts.

The variation in expression of a transgenic protein in a population is a well known phenomenon and reports have shown variations varying up to 100 fold or more (Peach and Velten, 1991; Mannerlof and Tenning, 1997). Inheritance patterns of transgenes also exhibit irregular behaviour, and frequently deviate from Mendelian expectations (Bregitzer *et al*., 1998; Bregitzer and Tonks, 2003). The control of transgene expression and silencing of transgenes has received particular attention in plants, because it reduces the reliability of transgenic approaches for genetic improvement of crops (Srinivasa Reddy *et al*., 2003) and the identification, selection and use of transgenic lines (Bregitzer and Tonks, 2003). The expression of transgenes can be unstable and is influenced by numerous factors (Hobbs *et al*., 1993; Matzke and Matzke, 1993; Finnegan and McElroy,

1994; Flavell, 1994, Matzke and Matzke, 1995; Kumpatla and Hall, 1998; Repellin *et al*., 2001; Bregitzer and Tonks, 2003; Srinivasa Reddy *et al*., 2003; Travella *et al*., 2005). For example, the introduction of multiple copies of transgenes into plants can lead to the loss of transgene expression, rather than an increase in expression (Mannerlof and Tenning, 1997). Moreover, both the transgene and homologous resident genes may be co-ordinately inactivated, a phenomena known as co-suppression (Matzke and Matzke, 1993; Finnegan and McElroy, 1994; Flavell, 1994; Brandle *et al*., 1995). These phenomena have been shown to occur with many different transgenes in a variety of plant species (Matzke and Matzke, 1993; Finnegan and McElroy, 1994; Flavell, 1994; Brandle *et al*., 1995). In addition, *in vitro* culture and regeneration protocols (particularly those involving the indirect route of morphogenesis) are known to induce genetic and epigenetic changes in plants, including gross chromosomal rearrangements, activation of dormant transposons, somaclonal variation and DNA methylation, which can affect gene expression (Peschke *et al*., 1987; Evans, 1989; Schmulders *et al*., 1995).

Previous studies have implicated various environmental factors in the appearance of transgene instability (Hart *et al*., 1992; Meyer *et al*., 1992; Dorlhac de Borne *et al*., 1994; Brandle *et al*., 1995; Neumann *et al*., 1997; Kohne *et al*., 1998). For example, Brandle and co-authors (1995) have reported that co-suppression of the *crs1-1* transgene for sulfonylurea herbicide resistance and the endogenous tobacco AHAS genes, *surA* and *surB* in field grown tobacco was triggered by the common agronomic practice of seedling transplantation. In Germany, a large field study of

petunia plants carrying a single copy of the maize A1 transgene demonstrated that stability of transgene expression was influenced by the age of the plant and environmental factors (Meyer *et al*., 1992). This petunia line showed considerably less variability in the greenhouse (Meyer *et al*., 1992). In a field study conducted in France, co-suppression of a nitrate reductase transgene and the endogenous tobacco genes was observed in up to 60% of homozygous field grown plants (Dorlhac de Borne *et al*., 1994). The appearance of this phenomenon in the greenhouse was delayed when the supply of light and/or nitrate was limiting. Since this study also involved transplantation of young plants, it was also possible that transplantation contributed to the appearance of co-suppression. Hart *et al*. (1992) observed that tobacco plants, carrying a chimeric chitinase gene, that were germinated and grown as seedlings in culture and subsequently transferred to soil, showed silencing of chitinase, whereas plants that had been directly seeded in soil in the greenhouse did not. The authors suggest that the *in vitro* conditions contributed to chitinase silencing.

In addition, transgene inactivation directly correlated to a heat treatment could be shown in transgenic *N. tabacum* plants (Neumann *et al*., 1997). After 10 days of incubation at 37^{o}C the plants lost the enzymatic activity encoded by the luciferase (*luc*) gene from *Photinus pyralis* while the *luc* mRNA steady state level was even increased. This strong reduction of the transgene-encoded activity was reversible and could be observed in up to 40% of the independent transgenic lines analyzed. Temperature sensitivity or stability seemed to depend on the DNA sequences surrounding the *luc* gene and not on its DNA sequence. The fact that the *npt II*

gene transferred to the plant on the same T-DNA seemed to undergo the same regulatory phenomenon led to the hypothesis that different transgenes might react in a similar manner to the heat treatment (Neumann *et al*., 1997).

That environmental factors affect the stability of transgene expression is also indicated in a study of transgenic cell suspension cultures of *Medicago sativa* carrying a single copy of an introduced herbicide resistance gene. A ten-day heat treatment of 37^{o}C resulted in loss of phosphinothricin resistance in 95% of cells from suspension cultures grown in non-selective medium. Loss of resistance in response to high temperatures was not due to loss of the introduced DNA, but rather to inactivation of the transgene (Walter *et al*., 1992).

Kohne and co-workers (1998) analyzed the influence of a heat-treatment on the expression of the *pat* herbicide resistance gene. After 10 days of cultivation at 37^{o}C, the herbicide resistance encoded by the chimeric *pat*41 gene (coding region from *Streptomyces viridochromogenes* fused to the 823 bp CaMV35S promoter) was strongly reduced in all of the 27 independent transgenic *Nicotiana tabacum* SRI lines. Neither the enzyme activity, the protein nor the *pat*41 specific RNA could be detected in the heat treated plants, regardless of the number of copies of the transgene and hemi- or homozygous states. In contrast to this, the expression of the synthetic *pat*S coding region fused to the 534 bp CaMV35S promoter and coding for essentially the same protein, was stable in heat treated plants. The exchange of the GC rich coding region of the *pat*41 gene by the AT rich synthetic

DNA fragment carrying the *pat*S coding region led to the stabilization of the specific RNA steady state level.

The stable expression of transgenic phenotypes is essential for the successful commercialisation of transgenic crops. A major condition for long term use of transgenic plant lines in breeding is the stable expression of the transgene once integrated into the plant genome. The transgene must be able to function under various environmental conditions in different genetic backgrounds and be stable over generations of breeding (Mannerlof and Tenning, 1997). From a breeder's aspect, it is favourable to have a single locus of the introduced character, in order to follow the new character as a single dominant Mendelian character during the breeding programme (Mannerlof and Tenning, 1997).

The objective of this study was to transform tobacco with the *pat* gene and evaluate the effects of subculturing and environmental stress on the stability of transgene expression in transgenic tobacco plants carrying the *pat* gene for resistance to the herbicide phosphinothricin.

2.2 Materials and methods

2.2.1 In vitro propagation of tobacco (*N. tabacum*) plants using axillary buds

Healthy *in vitro* propagated tobacco plants (*N. tabacum* cv. Samsun) were obtained from Dr. J.A. Brink (Agricultural Research Council, Roodeplaat, Pretoria, South Africa). These plants were propagated on Murashige and Skoog

(MS) medium (Murashige and Skoog, 1962) (Appendix A) containing 0.2 mg/ml kinetin (Sigma) (MSK), according to the method described by Draper *et al*. (1988). These *in vitro* grown plants of the same genotype were then used as a source of healthy leaves for the production of transformed tobacco leaf disks.

2.2.2 Transformation of tobacco leaf disks

Transformation of sterile tobacco (*N. tabacum* cv. Samsun) leaf disks was according to Draper *et al*. (1988) and McKenzie and Cress (1992). Working in a sterile petri dish, midribs of fully expanded young tobacco leaves obtained from *in vitro* grown plants were removed and the leaves cut into 0,5-1 cm² segments using a sterile scapel. Enough leaf tissue was processed for 10 petri dishes. The petri dishes were filled with 20 ml MS medium (Appendix A) containing 0.1 mg/l α-naphthaleneacetic acid (NAA) (Sigma) and 1 mg/l 6-benzyladenine (BA) (Sigma) (MSD4X2 medium) (Draper *et al*., 1998). Between 10 and 12 leaf disks were placed onto each of the 10 MSD4X2 plates, sealed with parafilm and incubated for two days at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 $\mu E\ m^{-2}\ s^{-1}$.

After two days incubation on MSD4X2 medium, tobacco leaf disks (non-transformed control leaf disks) from four of the MSD4X2 plates were placed onto 20 ml of the following media in duplicate: MSD4X2, MSD4X2 + 250 μg/ml cefotaxime (Sigma), MSD4X2 + 250 μg/ml cefotaxime + 100 μg/ml kanamycin (Boehringer Mannheim) and MSD4X2 + 100 μg/ml kanamycin.

The control (non-transformed) tobacco leaf disks on the various media above were placed at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1} and observed for the development of callus and shoots.

Transconjugate *A. tumefaciens* LBA4404 (Hoekema *et al*., 1983) containing pBI101 with the phosphinothricin acetyltransferase (*pat*) (obtained from the Agricultural Research Council, Roodeplaat, Pretoria, South Africa) and the neomycin phosphotransferase (*npt II*) genes was produced by Clint Viljoen (Viljoen *et al*., 1995) and was used for transformation experiments. The *npt II* marker gene allowed for kanamycin selection (Jefferson *et al*., 1987) and the *pat* gene for the herbicide, glufosinate ammonium (active ingredient is phosphinothricin–PPT) selection (Bayer *et al*., 1972). This transformed *Agrobacterium* was maintained at 30°C on 25 ml LB medium (Appendix B) containing 100 μg/ml rifampicin (Sigma) and 100 μg/ml kanamycin. *Escherchia* (*E*.) *coli*/pBI101/*pat* was maintained at 37°C on 25 ml LB medium (Appendix B) containing 100 μg/ml kanamycin and used for PCR and Southern blot analysis.

Leaf disks from the remaining six MSD4X2 plates were used for inoculation with the transconjugate *A. tumefaciens* culture containing the *pat* gene. The *Agrobacterium* culture was prepared by placing a loopful of freshly streaked transconjugate *Agrobacterium* into 10 ml YEP medium (Appendix B) containing 100 μg/ml rifampicin and 100 μg/ml kanamycin (to ensure maintenance of the plasmid) for 48 h at 30°C and then centrifuged at 2 000 *g* for 15 min. The broth

was decanted and the pellet resuspended in 18 ml YEP medium (OD_{600}=0.6-0.8 nm) with and without 20 μM acetosyringone (Aldrich) (McKenzie and Cress, 1992).

Leaf disks were dipped into the transconjugate *A. tumefaciens* culture (with and without acetosyringone), the excess liquid shaken off and the leaf disks returned to the original MSD4X2 plates (for co-cultivation with the *Agrobacterium*), the plates sealed with parafilm and incubated at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1} for a further 3 days. Plates were observed daily for bacterial growth.

After three days incubation with *Agrobacterium*, before bacterial growth completely covered the leaf disks, the leaf disks were transferred to 20 ml of the following media, in duplicate, in order to select for growth of transformed plant cells and shoots: MSD4X2 + 250 μg/ml cefotaxime + 100 μg/ml kanamycin and MSD4X2 + 250 μg/ml cefotaxime. The plates were incubated at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1} and observed periodically for the development of callus and shoots (Draper *et al*., 1988).

As soon as shoots had developed from the transformed leaf disks within 10 days, they were transferred to 20 ml MS medium (Appendix A) containing 250 μg/ml cefotaxime, 100 μg/ml kanamycin and 0.5 mg/l PPT (Riedel-de-Haen); 20 ml MS medium containing 250 μg/ml cefotaxime, 100 μg/ml kanamycin and 1 mg/l PPT,

and 20 ml MS medium containing 250 μg/ml cefotaxime, 100 μg/ml kanamycin and 20 mg/l PPT, in order to select for transformed shoots which had rooted in the presence of kanamycin and PPT and therefore contained the functional *pat* gene. Shoots that developed from the non-transformed (control) leaf disks were transferred to MS medium without selection agents and to MS medium containing the varying concentrations of kanamycin and PPT (as above) for comparative purposes.

2.2.3 Re-establishment of cultured plantlets in soil

Transformed and non-transformed rooted tobacco shoots were gently removed from the medium when the shoot size was 3-5 cm, taking care not to damage the apex. The agar sticking to the roots was quickly washed off and the plantlet placed in a small plant pot (12 cm x 14.5 cm diameter) filled to the top with potting mixture (Appendix C). Roots were covered with potting mixture and firmed down gently while carefully supporting the plantlet. To avoid dessication, the potted plantlet was placed in a 100% humid environment by covering the plant with a glass jar. The plants were grown in a growth chamber at 25±2°C with a 12:12 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1} until acclimatized and were then grown further at 50% humidity. Plants were watered with Multifeed (Appendix C). Putative transgenic plants were assessed phenotypically (height of plant; shape, width and length of leaves; size, shape and colour of flowers). After flowering, seed was collected and stored in sealed envelopes in a cool, dry and contained area.

2.2.4 Propagation of transformed plants

After flowering and seeding, leaves from growth chamber-grown transformed plants were cut from the plants and sterilized for 15 min in 0.35 % (v/v) sodium hypochlorite and rinsed four times in sterile distilled water. The midvein was excised and the leaves cut into squares. Ten to twelve squares were placed onto 20 ml MSD4X2 medium containing 100 μg/ml kanamycin and 20 mg/l PPT for the production of callus and shoots. Shoots were then transferred to 20 ml MS medium (Appendix A) containing 100 μg/ml kanamycin and 20 mg/l PPT to induce rooting (Draper *et al*., 1988). Transformed plantlets (shoot height 3-5 cm) were transferred to potting mixture as described above (2.2.3) and grown in the growth chamber at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}.

2.2.5 Six cycles of propagation of *in vitro* grown transgenic tobacco plants to determine the stability of the *pat* gene *in vitro*

Three *in vitro* grown transgenic tobacco lines (resulting from one transformation) carrying the *pat* gene for resistance to PPT, were propagated and maintained for 14 months *in vitro* through six cycles of propagation using axillary buds to determine the stability of the *pat* gene *in vitro*. Each cycle of propagation took place after two months, or when the plants had reached the top of the tissue culture flasks. Once the plants had reached the top of the tissue culture flasks, they were removed and the stems cut into nodal sections, each containing an

axillary bud. The leaves from each section were trimmed off, leaving only a short (2-3 mm) petiole stump. One section was placed into a tissue culture flask (10 cm x 5 cm diameter) containing 30 ml MS medium (Appendix A) supplemented with 0.2 mg/ml kinetin, 20 mg/l PPT and 50 µg/ml kanamycin, so that the axil was level with the medium surface. These sections were incubated at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. Once one or more shoots had developed from the axillary bud (after 2-3 weeks), the axillary shoots were further propagated (or rooted) by excising them and then placing onto 30 ml MS medium (Appendix A) containing 20 mg/l PPT and 50 µg/ml kanamycin for the formation of roots. A non-transformed tobacco plant (control) was also propagated onto medium with herbicide and kanamycin for comparative purposes. The transformed plants were transferred to potting mixture as described above (2.2.3) and placed in the growth chamber for further growth at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. Phenotypic comparisons (height of plant; shape, width and length of leaves; size, shape and colour of flowers) were made with non-transformed tobacco plants. After flowering, seed was collected and stored in sealed envelopes in a cool, dry and contained area.

2.2.6 Total plant DNA extraction and PCR

To confirm integration of the *pat* gene in the genome of transformed tobacco plants after transformation and after each subculture, total DNA from leaves of non-transformed tobacco plants and new transformed shoots was isolated using the cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle,

1987). Isolation of pBI101/*pat* DNA from *E. coli*, which was to be used as a positive control, was performed using the method described by Sambrook *et al* (1989), Birnboim and Doly (1979) and Ish-Horowich and Burke (1981). The 1.3 kb chimeric gene construct to be used as a positive control was isolated from pBI101/*pat* and the DNA fragment was then purified from a 1% (w/v) low melting agarose (Sea Plaque) (FMC Products) gel (gel electrophoresis was for 2 ½ h at 80V at 4^{o}C) using the Agarose Gel DNA Extraction Kit (Boehringer Mannheim).

PCR was performed using *pat* gene specific primers which amplified the *pat* coding region, a 558 bp fragment, of the 1.3 kb chimeric gene construct (including the promoter, coding region and terminator). The PCR Core Kit (Boehringer Mannheim) was used for each PCR reaction. The PCR was carried out in a volume of 25 µl in a PCR tube in a thermal cycler.

Each reaction mixture contained 1X PCR reaction buffer [10 mM Tris-HCl, 50 mM KCL, pH 8.3 (20^{o}C)], 1.2 mM $MgCl_2$, 200 µM dNTP's (dATP, dCTP, dGTP, dTTP, pH 7.0), 0.5 µM of each of the 20 mer oligonucleotide primers (the sequences of the 20 mer oligonucleotide primers used were: left primer $^{5'}$ GTC TCC GGA GAG GAG ACC AG $^{3'}$ and right primer $^{5'}$ CCT AAC TGG CCT TGG AGG AG $^{3'}$) and 10 ng of template DNA. Sterile distilled water was added to give a total volume of 25 µl. The contents of the PCR tubes were mixed and centrifuged briefly at 13 000 *g*. The tubes were placed into the thermal cycler and the template DNA denatured at 95^{o}C for 5 min. The tubes were place on ice,

centrifuged briefly at 13 000 *g* and then 0.5 units of *Taq* DNA polymerase added. After mixing the contents of the tube and a brief centrifugation at 13 000 *g*, 25 µl of sterile mineral oil was added to the tube. The tubes were placed back into the thermal cycler and the DNA amplified by 30 repetitive cycles involving template denaturation (92^{o}C for 1 min), primer annealing (60^{o}C for 1 min) and extension (72^{o}C for 1 min). The DNA was then extended for 5 min at 72^{o}C. The reaction was stopped by placing the tubes on ice. Samples subjected to PCR included DNA from transformed tobacco plants, DNA from a non-transformed tobacco plant (negative control), water (negative control), pBI101/*pat* (positive control), 1.3 kb chimeric gene construct (positive control). PCR products were separated on a 0.8% agarose gel stained with ethidium bromide to detect the amplified 558 bp *pat* coding region. To demonstrate the efficient separation of the DNA fragment, the DNA molecular weight marker VI was used.

2.2.7 Southern blot analysis

Total nucleic acid was isolated from *E. coli* containing pBI101/*pat* as described previously (2.2.6). This DNA at a concentration of 1µg/µl was digested with *Eco* R1 (Boehringer Mannheim) for 4 h at 37^{o}C and fractionated on a 0.8% (w/v) agarose gel by electrophoresis. Genomic DNA was extracted from leaves of transformed tobacco plants 1, 2 and 3, which tested positive for *pat* by PCR as previously described (2.2.6). DNA was also extracted from leaves of non-transformed tobacco plant which served as a negative control. A total of 10 µg of genomic DNA from the samples was digested with *Eco* R1 overnight (18 h) at

37°C. Samples were then fractionated on a 0.8% (w/v) agarose gel by electrophoresis. Samples fractionated included DNA from transformed tobacco, DNA from a non-transformed tobacco plant (negative control) and pBI101/*pat* (positive control). *Hind* III digested lambda-phage DNA (0,25 µg/µl) (Boehringer Mannheim) was used as a molecular marker. After electrophoresis, Southern blot hybridization was carried out according to Sambrook *et al*. (1989). DNA samples were blotted onto a positively charged nylon membrane (Boehringer Mannheim) with 20X SSC (Appendix D) according to the manufacturer's recommended procedure. Preparation of the probe was as follows: pBI101/*pat* was subjected to PCR and the 558 bp *pat* coding region was confirmed by fractionating on a 0.8% agarose gel. The DNA fragment was then purified from a 1% (w/v) low melting agarose (Sea Plaque) (FMC Products) gel (gel electrophoresis was for 2 ½ h at 80V at 4°C) using the Agarose Gel DNA Extraction Kit (Boehringer Mannheim) and labelled with digoxigenin using the DIG DNA Labeling Mix (Boehringer Mannheim).

The blot was hybridized overnight with the digoxigenin-labeled DNA probe at 68°C in DIG Standard Hybridization buffer containing 50% formamide (Boehringer Mannheim). After hybridization, the blot was washed twice for 5 min each in 2X SSC + 0.1% SDS at room temperature, followed by two washes with 0.1X SSC +0.1% SDS for 15 min at 68°C under constant agitation. Chemiluminescent detection was performed with CDP-Star (Boehringer Mannheim) according to the manufacturer's instructions.

2.2.8 Progeny analysis

Transgenic tobacco plants produced seed (T1) that was collected and planted in potting mixture (Appendix C). These seeds germinated in the potting mixture. Seedlings grew in the growth chamber at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. Total plant DNA was extracted as described above (2.2.6) and PCR performed (2.2.6) to determine the presence of the *pat* gene in the T1 plants. T1 plants produced seed (T2) and T2 plants produced seed (T3) that was collected and planted in potting mixture. Total plant DNA was extracted as described above (2.2.6) and PCR performed (2.2.6) to determine the presence of the *pat* gene in the progeny. Total plant DNA was also extracted from non-transformed control DNA for comparative purposes. Phenotypic (height of plant; shape, width and length of leaves; size, shape and colour of flowers) comparisons of the T1, T2 and T3 plants were made with non-transformed tobacco plants.

2.2.9 Spraying of transgenic tobacco plants

Twenty transformed tobacco parent plants, 20 T1 transformed plants and 20 non-transformed control tobacco plants grown to a height of 40 cm in the growth chamber at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. These plants were sprayed with a 2% aqueous solution of the commercial herbicide Basta (containing 200 g/l of the active ingredient, PPT) (Hoechst AG) (De Block *et al.*, 1987). The plants were

uniformly sprayed from approximately 20 cm distance using a normal household spray.

2.2.10 High temperature and drought stress experiment

Transgenic tobacco plants grown in the growth chamber were subjected to temperatures of 30°C, 35°C and 40°C and drought stress, to determine whether these environmental conditions had an effect on expression of the *pat* gene. Twelve transgenic tobacco plants (of the same genotype) of similar size and developmental stage (5-7 leaf stage) were used for each temperature tested. The temperature of the growth chamber was changed for each temperature experiment. Six transgenic tobacco plants were subjected to drought stress by limiting the supply of water (with Multifeed) to 50 ml every 3 days. This resulted in reversable wilting of the lower half of the leaves. The other six transgenic plants were kept under well-watered conditions (once a day with 300 ml of water) at the three temperatures tested. Two non-transformed tobacco plants were used as negative controls. When the leaves of the drought stressed tobacco plants had wilted, leaf material was harvested from all tobacco plants and the *pat* protein (phosphinothricin acetyltransferase - PAT) detected and quantified using a PAT-ELISA kit (Steffens, Biotechnische Analysen, GmbH). A standard curve was included on each ELISA plate in dilutions using 0, 0.40, 0.66, 1.1, 1.8, 2.9, 4.9 and 8.0 ng PAT/ ml. The standards were intended to check the assay performance and to provide quantitative results. The drought stressed tobacco plants were allowed to recover (watered twice a day with 300 ml of water containing Multifeed) at the three temperatures tested and then another ELISA was

performed on the leaves to assess expression of the *pat* gene. Averages were calculated to get ng PAT/ml.

To confirm the presence of the *pat* gene in these transgenic tobacco plants after the temperature and drought experiments, leaves were cut from the plants and sterilized for 15 min in 0.35 % (v/v) sodium hypochlorite and rinsed four times in sterile distilled water. The midvein was excised and the leaves cut into squares. Ten to twelve squares were placed onto 20 ml MSD4X2 medium containing 100 µg/ml kanamycin and 20 mg/l PPT for the production of callus and shoots. Shoots were then transferred to 20 ml MS medium containing 100 µg/ml kanamycin and 20 mg/l PPT to induce rooting. Transformed plantlets (shoot height 3-5 cm) were transferred to potting mixture as described above (2.2.3). Plants were grown in the growth chamber at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. PCR analysis was then performed on these plants to confirm presence of the *pat* gene (2.2.6).

2.3 Results

2.3.1 *In vitro* propagation of tobacco plants using axillary buds

Healthy tobacco plants (*N. tabacum* cv. Samsun) of the same genotype were propagated according to the method described by Draper *et al*. (1988) and successfully grown *in vitro* on MSK medium. These *in vitro* grown plants were

then used as a source of healthy leaves for the production of transformed tobacco leaf disks.

2.3.2 Transformation of tobacco leaf disks, maintenance and propagation of cultured plantlets

All non-transformed (control) leaf disks grown on MSD4X2 medium and MSD4X2 medium containing 250 µg/ml cefotaxime grew rapidly and produced callus as well as numerous shoots within ten days (Fig. 2.1). All non-transformed leaf disks placed onto MSD4X2 medium containing 250 µg/ml cefotaxime and 100 µg/ml kanamycin, and MSD4X2 medium containing only 100 µg/ml kanamycin, did not produce any shoots or callus and within 21 days these leaf disks showed signs of chlorosis and became necrotic (Fig. 2.2). These non-transformed leaf disks were therefore susceptible to kanamycin that had an inhibitory effect on leaf disk survival and growth.

The sterile leaf disks obtained from the *in vitro* grown plants were cut into 0,5-1 cm^2 pieces and inoculated with the *A. tumefaciens* transconjugate as described (2.2.2). Leaf disks inoculated with *A. tumefaciens* transconjugate were placed onto MSD4X2 medium containing 250 µg/ml cefotaxime and 100 µg/ml kanamycin and MSD4X2 medium containing only 250 µg/ml cefotaxime. All these leaf disks on the latter medium (containing cefotaxime only) produced shoots and callus within 15-21 days. All transformed leaf disks placed onto medium containing cefotaxime and kanamycin, developed callus and shoots after

Figure 2.1: Non-transformed (control) tobacco leaf disks grown on MSD4X2 medium containing 250 µg/ml cefotaxime. Callus (c) and shoots (s) were produced within ten days (*bar* 10 mm).

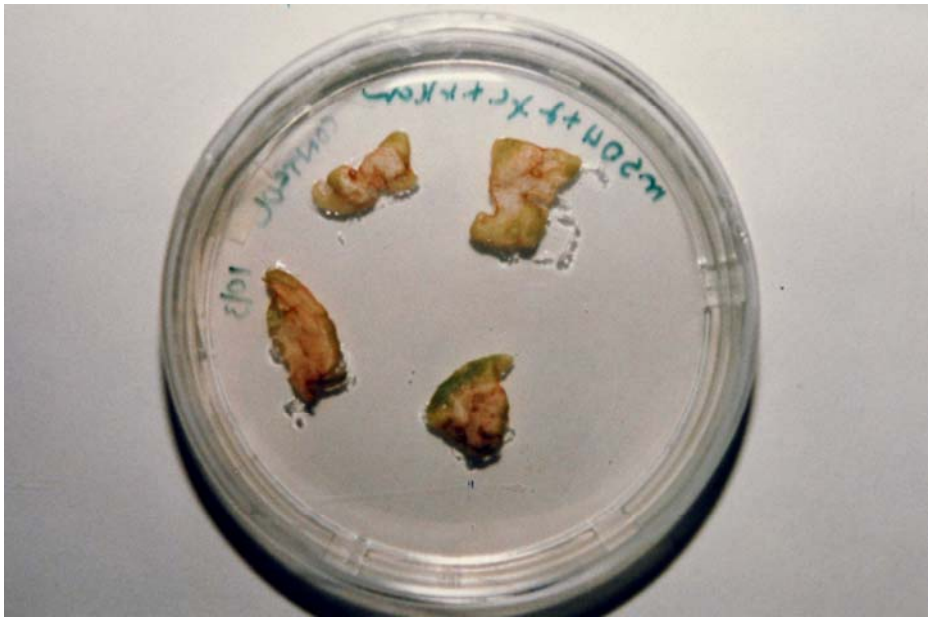

Figure 2.2: Non-transformed tobacco leaf disks on MSD4X2 medium containing 250 µg/ml cefotaxime and 100 µg/ml kanamycin. No callus or shoots were produced and within 21 days the leaf disks showed signs of chlorosis and became necrotic (*bar* 10 mm).

21 days. In some cases rooting was observed. The shoots and callus were considerably smaller and fewer in number than those seen on the inoculated disks placed onto cefotaxime plates only. All transformed leaf disks inoculated with acetosyringone developed callus and shoots more rapidly (within 15 days) (Fig. 2.3) than those transformed in the absence of acetosyringone (callus and shoots produced after 21 days).

After two months, shoots from the non-transformed leaf disks on MSD4X2 medium were placed onto MS medium as well as onto MS medium containing 100 μg/ml kanamycin and different concentrations of PPT (0.5, 1, 20 mg/l), to induce rooting. Every week for four weeks, the shoots were transferred to fresh medium. After four weeks, all non-transformed shoots exposed to kanamycin and all concentrations of PPT had become chlorotic and died without rooting (Fig. 2.4). In contrast, all non-transformed shoots on MS medium without selective agents, rooted within two weeks (Fig. 2.5).

Shoots from the transformed explants on MSD4X2 medium containing kanamycin and cefotaxime were placed onto MS medium containing 250 μg/ml cefotaxime, 100 μg/ml kanamycin and PPT (0.5, 1 and 20 mg/l) to induce rooting. New growth of leaves and expansion of leaves occurred (Figs. 2.6 and 2.7), compared to the non-transformed shoots that had died (Fig. 2.4). All shoots from the transformed leaf disks also rooted on all concentrations of PPT within one week as seen in Figs. 2.6 and 2.7. Roots formed highly branched rooting systems on the medium. In contrast, non-transformed shoots did not produce new shoots

Figure 2.3: Tobacco leaf disks, transformed with transconjugate *Agrobacterium*, in the presence of acetosyringone, produced callus (c) and shoots (s) within 15 days on MSD4X2 medium containing 250 µg/ml cefotaxime and 100 µg/ml kanamycin (*bar* 10 mm).

Figure 2.4: Non-transformed (control) tobacco shoots on MS medium containing 100 µg/ml kanamycin, 250 µg/ml cefotaxime and 20 mg/l PPT. The shoots showed signs of chlorosis and become necrotic (*bar* 10 mm).

Figure 2.5: Non-transformed tobacco shoots on rooting medium with no selection agents. Rooting (r) occurred within two weeks (*bar* 10 mm).

Figure 2.6: Transformed tobacco shoots on MS medium containing 100 µg/ml kanamycin, 250 µg/ml cefotaxime and 0.5 mg/l PPT. These transformed shoots produced roots within one week (*bar* 10 mm).

Figure 2.7: Transformed tobacco shoots on MS medium containing 100 µg/ml kanamycin, 250 µg/ml cefotaxime and 20 mg/l PPT. These transformed shoots produced roots (r) within one week (*bar* 10 mm).

Figure 2.8: A month old transformed tobacco plant rooting and growing on MS medium containing 100 µg/ml kanamycin, 250 µg/ml cefotaxime and 20 ml/PPT (*bar* 10 mm).

or roots in the presence of the different concentrations of PPT (Fig. 2.4). From these results it would appear that the *pat* gene was functional in the transformed tobacco shoots and was successfully detoxifying the herbicide.

After a month on the MS medium containing 100 μg/ml kanamycin and 20 mg/l PPT, nine transformed tobacco shoots which had developed new leaves and a good rooting system (Fig. 2.8) were transferred to pots containing potting mixture and placed into the growth chamber for further growth at 25°±2°C with a 12:12 h light:dark photoperiod. These plantlets adapted to the new environment and continued to grow into mature plants (Fig. 2.9) that flowered and produced seed. Nine potential transgenic tobacco plants were obtained. The nine transformed plants did not differ in any way phenotypically (height of plant; leaf shape, width and length; size, shape and colour of flower) from the non-transformed (control) plants (Fig. 2.10). This indicated that the inserted *pat* gene did not have any detrimental effect on the morphology of the transformed tobacco plants. The transformed plants flowered normally and set seed.

Propagation of transformed plants by the leaf disk method was successful, and many transformed plants were available for further studies. PCR and Southern blotting was performed on the nine transformed plants to confirm integration of the *pat* gene in the plant genome. PCR confirmed the presence of the *pat* gene in all nine transgenic tobacco plants. The PCR results for three transgenic tobacco lines (1, 2, 3) are shown (Fig. 2.11). A 558 bp *pat* coding region was seen for all three transgenic tobacco plants (Fig. 2.11, lanes 6-8). This indicated the presence

Figure 2.9: A healthy two month old transformed tobacco plant in soil.

Figure 2.10: A transformed tobacco plant (left) and a non-transformed tobacco plant (right). The transformed plant does not differ phenotypically from the control plant (*bar* 11.5 mm).

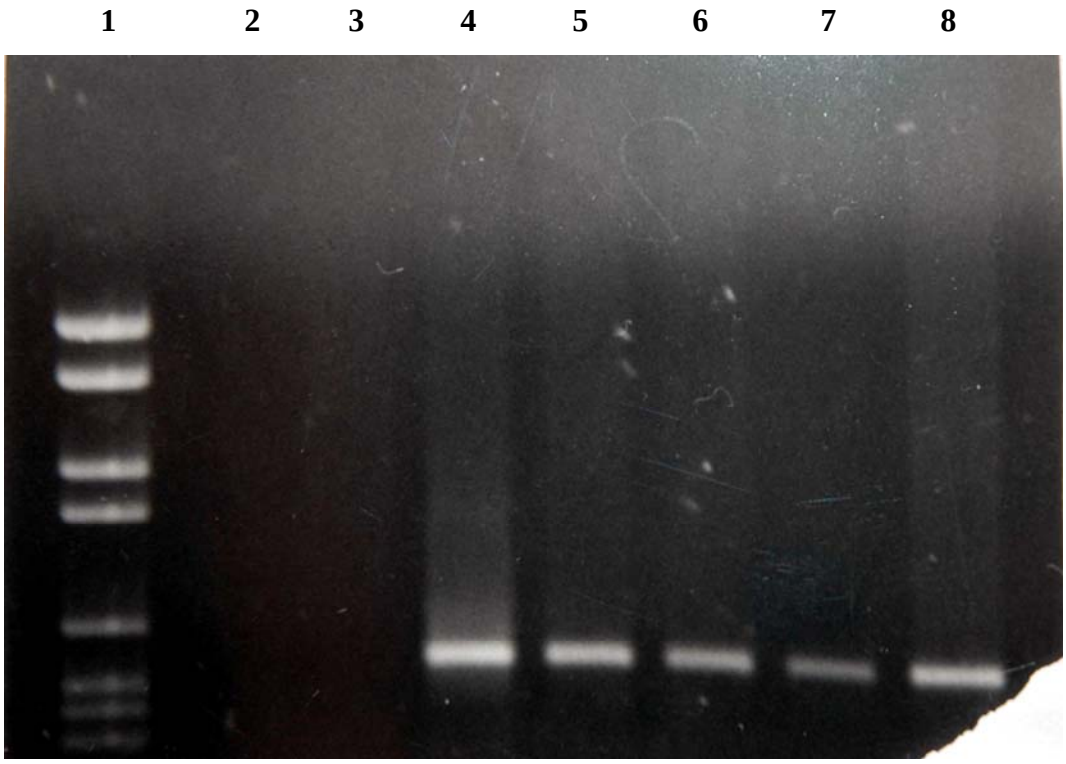

Figure 2.11: Amplification of the 558 bp *pat* coding region in 3 transgenic tobacco lines (1, 2, 3). Samples of 25 µl were subjected to 30 cycles of amplification. Two microlitres of each sample were resolved on a 0.8% agarose gel and visualized by ethidium bromide fluorescence.

Lanes (1) molecular weight marker VI; (2) negative control - water sample; (3) negative control – DNA from a non-transformed tobacco plant; (4) positive control - pBI101/*pat* DNA; (5) positive control - 1.3 kb chimeric gene construct; (6-8) DNA from transgenic tobacco lines 1-3.

of the *pat* gene in the genome of the three transgenic tobacco plants. No bands were seen in the negative water control (Fig. 2.11, lane 2) and DNA from the non-transformed tobacco plant (Fig. 2.11, lane 3), since only DNA segments with sequence information available for the primers could be specifically amplified. A 558 bp fragment was seen in the positive pBI101/*pat* DNA control (Fig. 2.11, lane 4) and for the 1.3 kb chimeric gene construct (Fig. 2.11, lane 5), as expected. No "primer dimers" (amplification artefacts) were observed, indicating that the PCR reaction was efficient and optimized. DNA from a transformed tobacco plant and DNA from a non-transformed tobacco plant restricted with *Eco* R1 showed smears on the agarose gel, indicating successful and complete restriction (Fig. 2.12, lanes 3-6). When Southern blotting was performed, DNA from all nine transformed tobacco plants showed no detectable hybridization signals even though these plants were positive for PCR-based tests and *in vitro* tests for resistance to PPT.

Results are only shown for three transgenic tobacco lines (1, 2, 3) (Fig. 2.13, lanes 4-6). The labelled probe bound to the positive pBI101/*pat* DNA control as seen in Fig. 2.13, lane 2. As expected, no band was observed for the non-transformed tobacco (negative control; Fig. 2.13, lane 3).

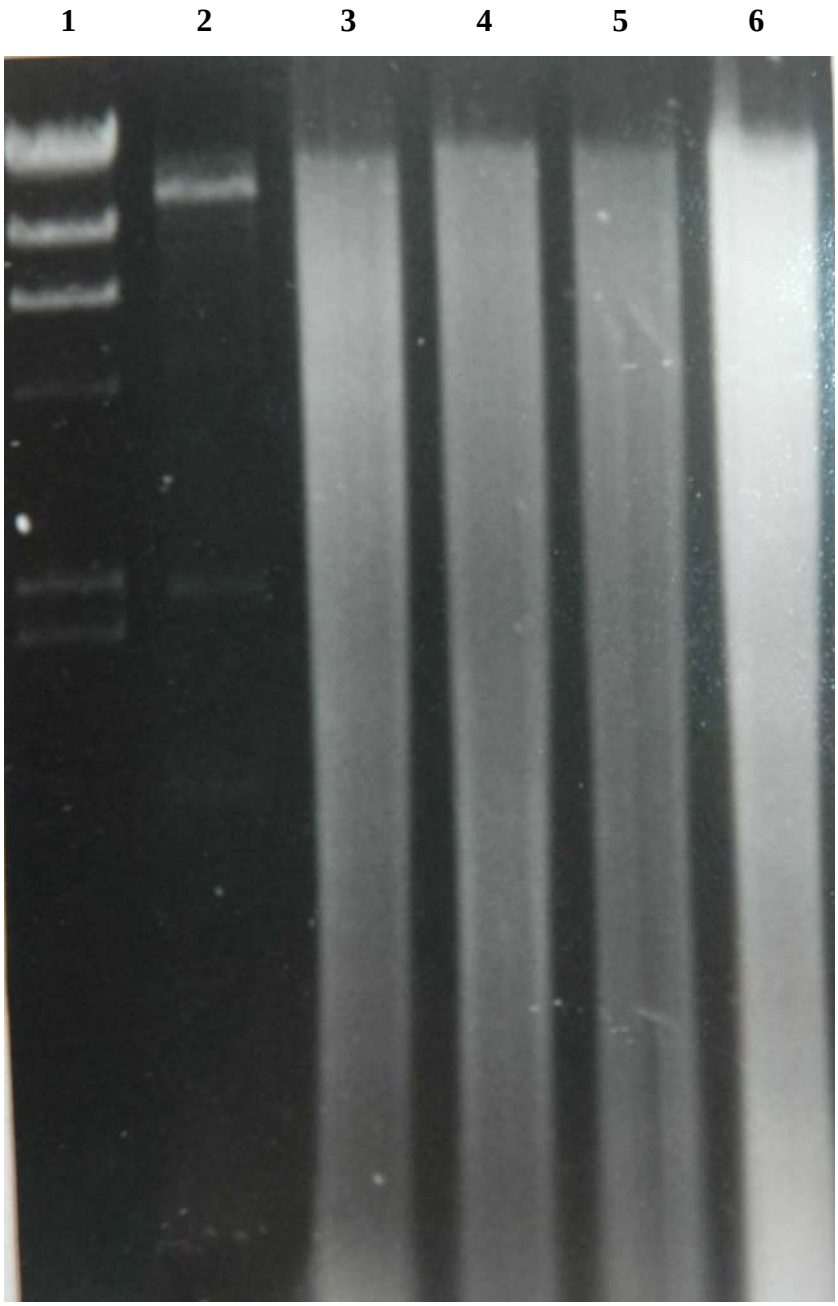

Figure 2.12: A 0.8% agarose gel used in Southern blot analysis to detect the presence of the *pat* gene in transformed tobacco DNA. DNA from non-transformed and transformed tobacco lines 1, 2, 3 (which tested positive for PCR) was restricted with *Eco* R1 and subjected to electrophoresis.

Lanes: (1) *Hind* III digested lambda DNA molecular weight marker; (2) *Eco* R1 restricted pBI101 /*pat* DNA; (3) restricted DNA from non-transformed tobacco plant; (4-6) restricted DNA from transformed tobacco lines 1-3.

Figure 2.13: Autoradiograph of the Southern blot analysis performed to detect the presence of the *pat* gene in the DNA of transformed tobacco lines 1, 2, and 3.

Lanes: (1) *Hind* III digested lambda DNA molecular weight marker; (2) *Eco* R1 restricted pBI101/*pat* DNA; (3) restricted DNA from a non-transformed tobacco plant; (4-6) restricted DNA from transformed tobacco lines 1-3.

2.3.3 Six cycles of propagation of *in vitro* grown transgenic tobacco plants to determine the stability of the *pat* gene *in vitro*

The three *in vitro* grown transgenic tobacco lines carrying the *pat* gene for resistance to the herbicide PPT were propagated six times onto medium containing 20 mg/l PPT and 50 μg/ml kanamycin to determine the stability of the *pat* gene *in vitro*. Within one week, axillary shoots developed, and after 3-4 weeks, these shoots were excised and placed onto fresh medium for root development. All the shoots developed roots and whole plants were formed on medium containing 20 mg/l PPT and 50 μg/ml kanamycin. Control (non-transformed) tobacco plants died on this medium.

The presence of the 558 bp *pat* coding region in all three transgenic tobacco lines after each cycle of propagation was confirmed by PCR. The results presented are the PCR results after the 6th *in vitro* propagation (Fig. 2.14, lanes 6-8). No 558 bp fragment was present in the DNA from the non-transformed tobacco plant (Fig. 2.14, lane 3), as expected. The transformed plants which were propagated six times on selection medium showed no phenotypic (height of plant; shape, width and length of leaves; size, shape and colour of flowers) differences when compared with control plants grown on medium without kanamycin and PPT.

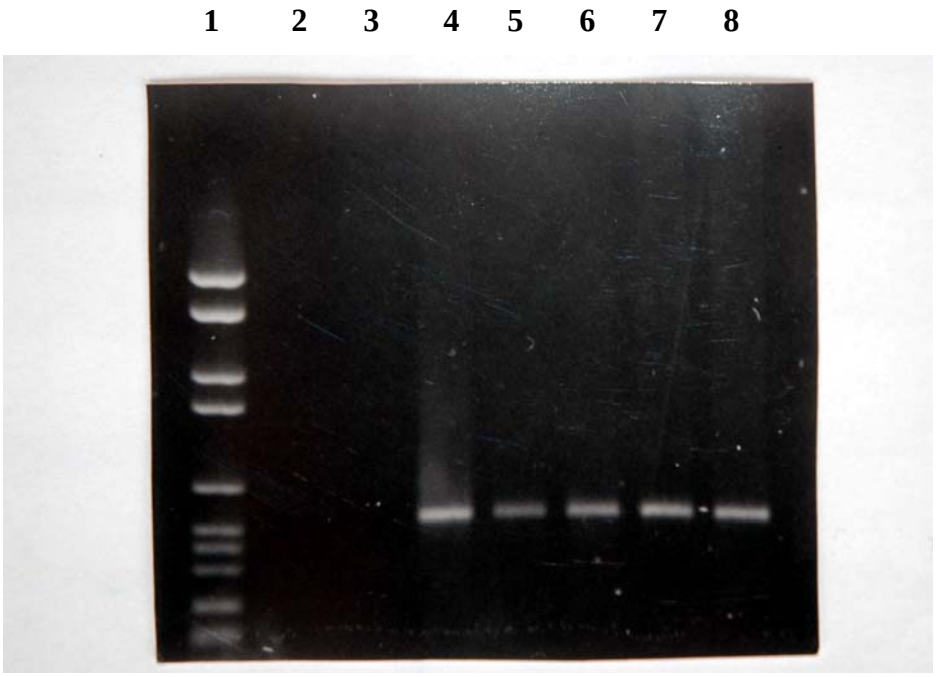

Figure 2.14: Amplification of the 558 bp *pat* coding region in 3 transgenic tobacco lines after the 6th *in vitro* subculture. Samples of 25 µl were subjected to 30 cycles of amplification. Two microlitres of each sample were resolved on a 0.8% agarose gel and visualised by ethidium bromide fluorescence.

Lanes: (1) molecular weight marker VI; (2) negative control - water sample; (3) negative control – DNA from a non–transformed tobacco plant; (4) positive control - pBI101/*pat* DNA; (5) positive control - 1.3 kb chimeric gene construct; (6-8) DNA from transgenic tobacco lines 1-3.

2.3.4 Progeny analysis

The 558 bp *pat* coding region was present in T1, T2 and T3 plants that were grown in the growth chamber (Fig. 2.15, lanes 6-8). No 558 bp fragment was present in the DNA from the non-transformed tobacco plant (Fig. 2.15, lane 3), as expected. A 558 bp fragment was present for the positive controls, pBI101/*pat* and 1.3 kb chimeric gene construct (Fig. 2.15, lanes 4 and 5, respectively), as expected. There were no phenotypic (height of plant; shape, width and length of leaves; size, shape and colour of flowers) differences between T1, T2 and T3 progeny when compared with DNA from the non-transformed tobacco plant.

2.3.5 Spraying of transgenic tobacco plants

The 20 non-transformed plants showed necrotic lesions within 48 hours and after 4-7 days all non-transformed tobacco plants died. The 20 T0 and 20 T1 tobacco plants that were transformed remained green and were fully tolerant to the herbicide after two weeks (Fig. 2.16). The application of the herbicide did not affect growth of the transgenic plants and treated plants flowered normally and set seed.

2.3.6 High temperature and drought stress experiment

The presence of *pat* gene in 18 transgenic tobacco plants that were subjected to temperatures of 30^{o}C, 35^{o}C and 40^{o}C and 18 transgenic tobacco plants that were

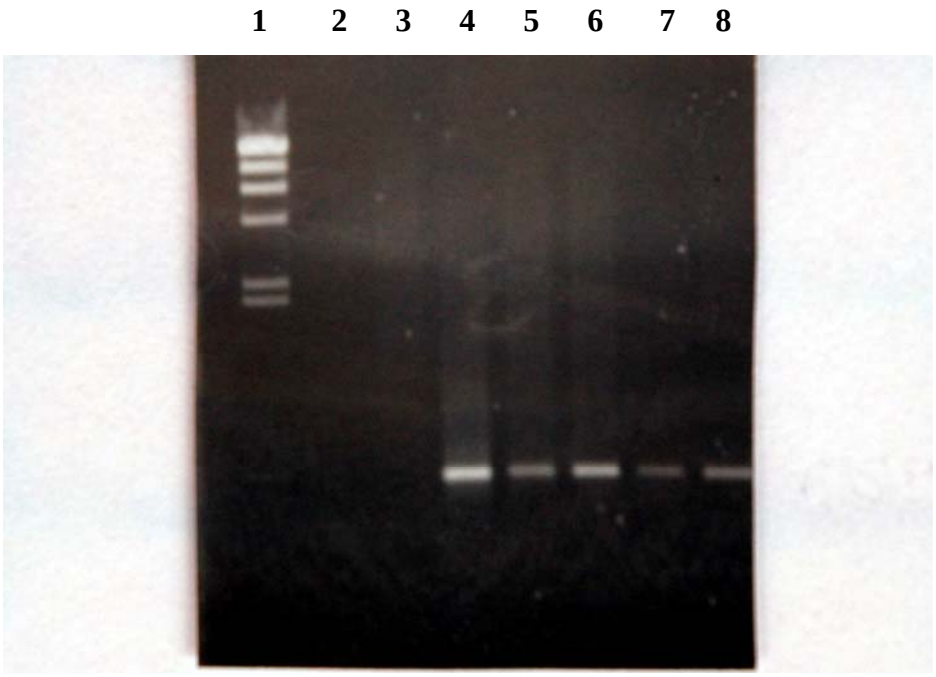

Figure 2.15: Amplification of the 558 bp *pat* coding region in T1, T2 and T3 tobacco progeny. Samples of 25 µl were subjected to 30 cycles of amplification. Two microlitres of each sample were resolved on a 0.8% agarose gel and visualised by ethidium bromide fluorescence.

Lanes: (1) molecular weight marker VI; (2) negative control - water sample; (3) negative control – DNA from a non–transformed tobacco plant; (4) positive control - pBI101/*pat* DNA; (5) positive control - 1.3 kb chimeric gene construct; (6-8) DNA from T1, T2 and T3 tobacco progeny.

Figure 2.16: Spraying of transformed tobacco plants with PPT. Non-transformed tobacco plants died (left and middle) and the transformed tobacco plant survived (*bar* 10 mm).

drought stressed at 30°C, 35°C and 40°C, was determined by the PAT-ELISA. All leaf material samples from all the transgenic plants developed a blue colour indicating that all the plants were producing PAT (Fig. 2.17). The non-transformed leaf material from the control plants did not produce a blue colour as expected, since they did not contain the *pat* gene. The results of PAT detection in the non-stressed and drought stressed transgenic tobacco plants at 30°C, 35°C and 40°C are shown in Table 2.1. When the drought stressed plants were allowed to recover, the amount of PAT produced by the recovered plants did not differ from when they were stressed (7.6, 7.6, 8.0 ng PAT/ml at temperatures 30°C, 35°C and 40°C, respectively).

When leaves of the transgenic tobacco plants, after subjection to high temperature or drought stress, were cultured on medium containing 100 µg/ml kanamycin and 20 mg/l PPT, shoots were produced. All these shoots then rooted in medium containing 100 µg/ml kanamycin and 20 mg/l PPT, indicating that the *pat* gene was still stably integrated in the tobacco plants. PCR confirmed the presence of the *pat* gene in these tobacco plants. A 558 bp fragment was seen for all 3 transgenic tobacco plants. This indicated the presence of the *pat* gene in the genome of these transgenic plants. No bands were seen in the negative water control and DNA from the non-transformed tobacco plant, since only DNA segments with sequence information available for the primers could be specifically amplified. A 558 bp fragment was seen in the positive pBI101/*pat* DNA control and for the 1.3 kb chimeric gene construct, as expected.

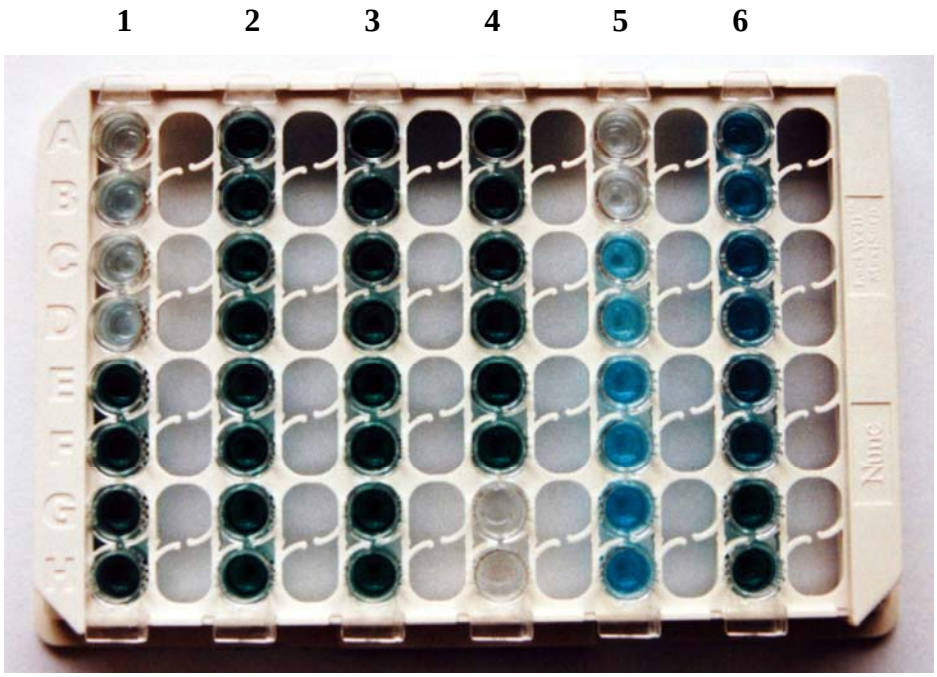

Figure 2.17: Detection of PAT in environmentally stressed transgenic tobacco plants using the *pat*-ELISA kit.

Lanes: (1) a-d: non-transformed tobacco; e-h: transgenic tobacco at 30°C; (2) a-h: transgenic tobacco at 30°C; (3) a-h: transgenic tobacco at 30°C and drought stressed at 25°C; (5) a-h: PAT standards – 0, 0.40, 0.66, 1.1 ng PAT/ml; (6) a-h: PAT standards – 1.8, 2.9, 4.9, 8 ng PAT/ml.

Table 2.1: The amount of PAT produced when transgenic plants were non-stressed and drought stressed at 30^{o}C, 35^{o}C and 40^{o}C.

	ng PAT/ml		
Temp (^{o}C)	Control	non-stressed	drought stressed
25	7.5		
30		7.7	7.5
35		7.6	7.7
40		8.0	8.0

2.4 Discussion

2.4.1 *In vitro* propagation of tobacco (*N. tabacum*) plants using axillary buds

Plant propagation is a useful way of providing a uniform supply of uncontaminated, genetically identical plant/shoot cultures for transformation experiments. The generation of plant material by propagation *in vitro* does have the advantage that numerous cultures of cells of the same genotype are produced to study transformation events. The plants are also kept free from insect and microbial pests. It does, however, involve more work and is more costly than simply germinating sterile seeds *in vitro* when required or sterilizing explants from greenhouse-grown seedlings. The technique of plant propagation *in vitro* depends on the species under consideration. Most propagation methods for dicotyledonous plants involve the use of axillary bud explants (Draper *et al*., 1988). The method used for the propagation of tobacco plants using axillary buds in this study and in other previous studies involving tobacco (Draper *et al*., 1988) was successful and provided a source of healthy leaves for the production of transformed tobacco. Tobacco has been an excellent 'model' system since the 1970s. MSK is a typical propagation medium (Draper *et al*., 1988) and the low level of kinetin helped to maintain the vigour of the explant and stimulate the development of axillary shoots from preformed quiescent axillary buds in this study.

2.4.2 Transformation of tobacco leaf disks, maintenance and propagation of cultured plantlets and progeny analysis

Transformation of tobacco was achieved by leaf disk transformation with the transconjugate *A. tumefaciens* strain. Co-cultivated leaf disks placed onto medium containing kanamycin and PPT produced callus and shoots that rooted. In contrast, non-transformed leaf disks became necrotic within 21 days on the above medium. These results are in keeping with the results of other researchers (Wohlleben *et al*., 1988) who reported that transformed tobacco leaf disks with the *pat* gene produced shoots and rooted in the presence of PPT and which were indistinguishable from non-transformed plants grown on herbicide-free medium. The wounded edges were susceptible to infection with the culture and were sites of rapid cell division and induction of shoot regeneration (Horsch *et al*., 1985; Sunilkumar *et al*., 1999).

From the results obtained in this study, it was demonstrated that tobacco plants transformed with the *pat* gene were resistant to the herbicide glufosinate ammonium (active ingredient, PPT). It was shown that the non-transformed tissues, such as the leaf disks and shoots were sensitive to a very low dose of PPT (0.5 mg/l) and that the transgenic plants carrying the modified *pat* gene were able to grow on medium with 20 mg/l PPT. Similarly, Wohlleben *et al*. (1988) found that transgenic tobacco plants carrying a modified *pat* gene were able to grow on medium with at least 50 mg/l PPT and non-transformed tissues were sensitive to a very low dose of the herbicide (less than 1 mg/l). It has been reported that

tobacco calli expressing the *bar* gene could proliferate on medium containing 500 mg/l PPT (De Block *et al*., 1987). The ability of a shoot to root on selection medium containing high levels of selection agents (as was done in the present study) is a very strong indication of its transformed nature, as in general, differentiated structures such as shoots often tend to be naturally less sensitive to selection agents than callus. The increase of selection pressure (0.5-20 mg/l PPT) in this study had no definite influence on shoot regeneration or rooting, suggesting that once cells were transformed they were resistant to higher doses of PPT. Similar results have been shown in the study done by Akama *et al*. (1995).

The length of time of submergence of the leaf disks in the bacterial culture in this study allowed for transformation of the leaf disks and regeneration was not impaired.

Induction of *vir* genes can be achieved by acetosyringone and alpha-hydroxyacetosyringone (Stachel *et al*., 1985) and also *in vitro* by co-cultivation of *Agrobacterium* with the wounded plant cells or tissues or in media containing defined signal molecules such as acetosyringone (Godwin *et al*., 1991). Other researchers have demonstrated that effective *vir* induction requires a medium with pH less than 5.7, a carbon source such as sucrose and a temperature below 30°C (Stachel *et al*., 1985).

The inclusion of acetosyringone in the bacterial culture medium used for the transformation of tobacco leaf disks in this study allowed for transfer of the *pat*

gene into the plant genome and promoted the production of transformed tobacco plants. The appearance of PPT-tolerant callus and shoots on tobacco leaf disks inoculated in the absence of acetosyringone was slower (after 21 days) than the appearance of callus and shoots on those leaf disks inoculated in the presence of acetosyringone (within 15 days). The results in this study are similar to the results of the studies by Owens and Smigocki (1988), Godwin *et al*. (1991) and Lipp Joao and Brown (1993) who reported that by adding acetosyringone to inoculum, the transformation process is enhanced. Addition of acetosyringone to the co-cultivation has previously been shown to increase Ti transformation frequencies with tobacco (Godwin *et al*., 1991; Sunilkumar *et al*., 1999), tomato (Lipp Joao and Brown, 1993), broccoli (Henzi *et al*., 2000), cassava (Sarria *et al*., 2000) and soybean (Zhang *et al*., 1999a; Yan *et al*., 2000; Olhoft and Somers, 2001). In this study, there was 100% transformation of tobacco leaf disks. The most probable explanation is that acetosyringone treatment leads to an increased copy number of the inserted gene, presumably as a result of multiple insertions of the T-DNA into the host genome (Lipp Joao and Brown, 1993).

The existence and expression of the *pat* gene within the nine transgenic tobacco lines in the present study did not have any deleterious effects on growth characteristics, fertility or physical appearance of the plants. The transgenic plants appeared normal and their phenotype (height of plant; shape, width and length of leaves; size, shape and colour of flowers) resembled that of the regenerated control plants. The nine transgenic tobacco plants reached maturity and viable seeds were produced. These results are similar to the reports by Shah

et al. (1986) and Eckes *et al*. (1989), thus demonstrating that an enhanced level of an enzyme, combined with a method of genetic engineering, does not seem to affect the fitness of the tobacco plant.

Cultured shoots of transformed plants can often be propagated by one of two main routes. Shoots may be established in compost, rooted, grown photoautotrophically and the plants propagated by seed production. This of course is essential to study the normal expression and genetics of the transferred gene. However, a situation may arise where only one or a few transformed shoots are regenerated after a lot of hard work. In these cases, it is advisable to propagate this extremely valuable material vegetatively (Draper *et al*., 1988). In this study propagation of transformed plants by the leaf disk method was successful, and many transformed plants are available for further studies.

An essential proof of stable genetic transformation is the determination of integration of foreign DNA into the chromosome of the recipient plants. PCR was performed on transgenic tobacco and the presence of the *pat* gene was confirmed by the presence of the 558 bp fragment. Southern blot analysis, however, did not give evidence of integration of the *pat* gene in the transformed tobacco. Similarly, Sarria *et al*. (2000) reported that out of the three transformed cassava plants that tested positive for GUS expression, PCR-based tests and *in vitro* greenhouse tests for resistance to PPT, only one plant was positive for Southern-based tests of transgenics. The reason for no evidence of the integrated *pat* gene in the transformed tobacco plants by Southern blot analysis in this study could be due to

the low concentration of transformed DNA which was blotted. Insufficient labeling of the probe could also be a reason, since the plasmid control band was easily visible in the gel image of Fig. 2.12 and normally if this was present at a level comparable to a single copy gene in tobacco it would have been visible. The *pat* gene was present in the transformed tobacco- the ELISA tests were strongly positive for the PAT protein, the *pat* gene was present in all T1, T2 and T3 plants indicating integration of the *pat* gene in the tobacco nuclear genome and stable transmission of the *pat* gene over three seed generations.

Future studies could include more Southern blots with an efficiently labeled probe and more detailed progeny analysis (data on the number of tolerant and susceptible transgenic tobacco plants).

2.4.3 Cycles of propagation of *in vitro* grown transgenic tobacco plants to determine the stability of the *pat* gene *in vitro*

Stability of transgene expression is an important character of genetically modified plants. Transgene instability leading to silencing of the transgene and subsequent loss of the expected phenotype can result from various factors (Matzke and Matzke, 1993). In this study, I assessed whether six cycles of propagation of three transgenic tobacco plants over 14 months *in vitro* led to instability of the *pat* gene in the transformed tobacco plants. After each subculture, 100% of the axillary buds of the transgenic tobacco nodal sections produced shoots in the presence of the herbicide glufosinate ammonium (PPT). When all the new shoots were

excised and placed onto rooting medium containing the herbicide, 100% rooting occurred. This indicated successful integration of the *pat* gene in all the transgenic plants. The *pat* gene was producing the PAT enzyme that acetylated the free ammonium group of the herbicide glufosinate ammonium thereby detoxifying it (Murakami *et al*., 1986; De Block *et al*., 1987; Thompson *et al*., 1987) and allowing the transgenic nodal sections to produce shoots and roots in the presence of the herbicide. The non-transformed tobacco nodal sections did not produce shoots or roots in the presence of the herbicide and became necrotic. PCR of the DNA isolated from all three transgenic tobacco lines after each subculture resulted in a 558 bp fragment, indicating stable integration of the *pat* gene in the plant genome.

The six cycles of propagation of the tobacco plants by axillary bud multiplication was successful and provided a source of genetically stable tobacco plants for 14 months. For 14 months PAT enzyme expression was still present, indicating the stable integration of the *pat* gene in the plant genome. MS medium containing 0.2 mg/ml kinetin is a typical propagation medium for tobacco (Draper *et. al*., 1988) and the low level of kinetin helped to maintain the vigour of the explant and stimulate the development of axillary shoots from preformed quiescent axillary buds.

2.4.4 Spraying of transgenic tobacco plants

When the transgenic tobacco plants expressing *pat* were sprayed with PPT (equivalent to 20 l/ha Basta) in the greenhouse, they were all fully tolerant to PPT.

The control plants showed necrotic lesions within 48 hours. These results are similar to the results of other researchers who reported that control plants showed necroses within 48 hours and were killed within ten days by all herbicide treatments (de Greef *et al*., 1989). Forty eight hours after herbicide treatment in this study, the tolerant tobacco plants showed discolouration on parts of the leaves. This could be due to the herbicidal component itself, but it is more likely the consequence of the surfactants present in the commercial formulation of PPT, which is not adapted for selective use on crops (de Greef *et al*., 1989). The discoloration disappeared shortly afterwards. No visible effects or damage were further observed on the tolerant plants and they flowered normally and set seed. Similarly, De Block *et al*. (1987) reported that the growth of transgenic tobacco plants tolerant to the commercial application of PPT (equivalent to 20 l/ha Basta) was indistinguishable from non-transformed control plants and they flowered normally and set seed. Normal applications for weed control in agriculture vary from 2.5-7.5 l/ha (De Block *et al*., 1987).

2.4.5 High temperature and drought stress experiment

Previous studies have implicated various environmental factors in the appearance of transgene instability (Hart *et al*., 1992; Meyer *et al*., 1992; Walter *et al*., 1992; Dorlhac de Borne *et al*., 1994; Brandle *et al*., 1995; Neumann *et al*., 1997; Kohne *et al*., 1998). Drought is an important environmental factor limiting the productivity of crops worldwide. Many crops are grown in areas where rainfall is marginal or where drought stress is intermittent throughout the growing season

because soils have a low holding capacity. Heat is another stress often associated with drought.

The transgenic plants which were subjected to temperatures of 30^{o}C, 35^{o}C and 40^{o}C produced 7.7, 7.6 and 8.0 ng PAT/ml, respectively. When compared to 7.5 ng PAT/ml expressed by the control plants grown at 25^{o}C, it was evident that increased temperature had no adverse effect on the expression of the *pat* gene in the transgenic tobacco plants. At all temperatures tested the *pat* gene was stable and the protein detected by the ELISA. The drought stressed plants at the same temperatures also expressed the PAT protein and when the drought stressed tobacco plants were allowed to recover, the amount of PAT produced by the recovered plants did not differ from when they were stressed, indicating stable integration and expression of the *pat* gene. The temperatures tested as well as drought stress had no effect on the stable expression of the *pat* gene in the transgenic tobacco plants. This was confirmed by shoot and root regeneration from the tobacco leaves after heat and drought regimes on medium containing kanamycin and PPT and the 558 bp fragment visualized after PCR analysis.

The successful results obtained in this study on the stability of the *pat* gene in transgenic tobacco plants through six cycles of propagation, high temperature regimes and drought stress have set the platform for future studies on the stability of the *pat* gene in transgenic cotton and soybean.

2.5 Conclusions

The results of this study show that six cycles of propagation, temperatures of 30^{o}C, 35^{o}C and 40^{o}C and drought stress do not have an effect on the stable expression of the *pat* gene in transgenic tobacco plants. The *pat* gene was present in the T1, T2 and T3 progeny indicating successful transfer to progeny.

CHAPTER 3

The effect of various cytokinins on the induction of multiple shoots and plant regeneration in commercially grown South African soybean cultivars.

Sections of this chapter have been presented as a paper at the 24th Annual Conference of the South African Association of Botanists, University of Cape Town, South Africa, 12-16 January 1998.

C. McNaughton and M.E.C. Rey. Regeneration of South African soybean cultivars from shoot apex tissues for transformation.

This chapter will be submitted for publication in Plant Cell, Tissue and Organ Culture.

C. McNaughton-Pascoe and M.E.C. Rey. The effect of various cytokinins on the induction of multiple shoots and plant regeneration in commercially grown South African soybean cultivars.

Abstract

Efficient and reproducible plant regeneration protocols are essential for successful transformation of soybean (*Glycine max*). A rapid, simple, reproducible and possibly genotype independent method of *in vitro* propagation using shoot apical meristem explants has been developed for commercially grown South African soybean cultivars Talana and Ibis. Shoot apical meristems (0.4-1 mm in length) from 12 day old seedlings were cultured on MS medium supplemented with 0.0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.5 and 5.0 mg/l kinetin, zeatin, BA and TDZ, individually, and the formation of multiple shoots assessed after four weeks of culture at 25±2°C with a 16:8 h light:dark photoperiod. Talana and Ibis shoot apical meristems on media containing zeatin and kinetin caused loss of apex organization and the formation of green callus. Kinetin and zeatin did not induce multiple shoot formation from the shoot apical meristems. Shoot meristems from both cultivars differentiated into multiple green shoots on all concentrations of BA and TDZ tested. The highest percentage of Talana (62%) and Ibis (57%) shoot apical meristems producing healthy green shoots was on medium containing 0.05 mg/l BA. The number of shoots produced per meristem at this concentration was 6-7 for both Talana and Ibis. The percentage of Talana and Ibis shoot apical meristems producing multiple shoots was reduced at lower (0.01 mg/l) and higher (0.1-5 mg/l) concentrations of BA. The highest percentage of Talana (46%) and Ibis (39%) shoot apical meristems producing multiple shoots was on medium containing 1 mg/l TDZ. The number of shoots produced per meristem at this concentration was 6-7 for Talana and 5-6 for Ibis. The percentage of Talana and

Ibis shoot apical meristems producing multiple shoots was reduced at lower (0.01-0.5 mg/l) and higher (2.5 and 5 mg/l) concentrations of TDZ. Root initiation from the 300 shoots did not occur on half-strength MS medium, half-strength MS medium with hormones (0.05 mg/l and 0.1 mg/l NAA or IBA) or on B5 medium without hormones. MS medium containing 0.05 mg/l BA and 0.2 mg/l NAA was assessed for its potential in initiating and maintaining shoot formation from shoot apical meristems. Within one week, 100% of the meristems of Talana and Ibis differentiated into multiple green shoots (6-7 per meristem for both Talana and Ibis) accompanied by callus. Three hundred excised Talana and Ibis shoots (2-3 cm) were placed on B5 medium without hormones for root induction. After four weeks, shoots had elongated, more shoots proliferated and a well developed rooting system had appeared. The overall percentage of rooted shoots from the isolated shoots was 66% (198/300) for Talana and 52% (156/300) for Ibis. The regenerated plantlets (5-6 cm) were transferred to sterile potting mixture in tissue culture flasks under growth chamber conditions. After a month in the potting mixture, 100% plantlets developed new, green healthy shoots. Plantlets were transferred to sterile potting mixture in pots and acclimatized successfully in the growth chamber. These regenerated plants were phenotypically (height of plant; shape, width and length of leaves; size, shape and colour of flowers) normal when compared to seed-initiated plants, and set seed. Normal, fertile plants were therefore regenerated from Talana and Ibis shoot apical meristems. The overall frequency of regenerated soybean plants per meristem was 66% for Talana and 52% for Ibis. The shoot apical meristem-based regeneration method developed in this study can make a wider range of soybean germplasm accessible to

improvement either by particle bombardment or *Agrobacterium*-mediated gene transfer.

3.1 Introduction

Soybean [*Glycine* (*G*.) *max*] is a world crop, cultivated widely in the United States, Brazil, Argentina, China, India and South Africa (Singh and Hymowitz, 1999; Somers *et al*., 2003). Soybean is an economically important leguminous seed crop for high protein food and vegetable oil for human consumption, and of protein-rich livestock feed (Chee *et al*., 1989; Rech *et al*., 1989; Widholm *et al*., 1992; Singh and Hymowitz, 1999, Zeng *et al*., 2004). The demand for soybean, being the most produced, traded and utilized crop in the world has resulted in increased soybean production and research on this crop has also increased proportionally (Barwale and Widholm, 1990; Somers *et al*., 2003). The improvement and optimization of soybean characteristics, such as increasing resistance to pests and herbicides, and lowering allergenic protein levels in seeds, is therefore desirable.

Transformation techniques have produced new breeding materials that would not be available in the germplasm among cross-compatible species (Kaneda *et al*., 1997). Several transgenic soybeans have already been produced using *Agrobacterium*-mediated transfer (Hinchee *et al*., 1988; Parrot *et al*., 1989; Di *et al*., 1996; Zhang *et al*., 1999a; Clemente *et al*., 2000; Yan *et al*., 2000; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Ko *et al*., 2004; Zeng *et al*., 2004; Shan *et al*.,

2005), direct infection of germinating seeds with *Agrobacterium* (Chee *et al*., 1989; de Rhonde *et al*., 2001) and with DNA-coated microprojectiles (McCabe *et al*., 1988; Christou *et a*l., 1989; Finer *et al*., 1992; Sato *et al*., 1993; Moore *et al*., 1994; Parrot *et al*., 1994; Hadi *et al*., 1996; Stewart *et al*., 1996; Ponappa *et al*., 1999; Santarem and Finer, 1999; Aragao *et al*., 2000; Srinivasa Reddy *et al*., 2003). Transgenic plants have been obtained with each technique and the foreign genes inserted into these plants were stably inherited in the progeny of the transformed plants McCabe *et al*., 1988; Clemente *et al*., 2000; Yan *et al*., 2000; Olhoft *et al*., 2003; Zeng *et al*., 2004). Improvement in the regeneration frequency would contribute to an increase in the production of transgenic soybeans.

Soybean has been studied extensively in tissue culture, but despite these efforts, soybean and related species of this genus have been amongst the more difficult plant species from which to achieve regeneration of cells and plants from tissue cultures (Widholm *et al*., 1992). The major impediments to the development of a regeneration system for soybean have been genotype (because not all cultivars respond the same on regeneration medium) and the low rate of regeneration from explants. It has also been reported that grain legumes have been, in general, a lot more recalcitrant to *in vitro* manipulation than other species (Christou, 1997). Improvement of tissue culture methods to induce efficient regeneration in a genotype independent manner is desirable.

The first regeneration of soybean plants was reported by Kimball and Bingham (1973) where plants were regenerated from hypocotyl explants through shoot formation. Plant regeneration from somatic embryogenesis as a principle mode of regeneration was then described by Christianson *et al*. (1983) where plants were regenerated from a soybean suspension culture through embryogenesis at a low frequency from an immature embryo-derived culture. Lippmann and Lippmann (1984) induced somatic embryogenesis from cotyledons of immature embryos that did not develop further. Other more reliable embryogenic regeneration systems have been described by Lazzeri *et al*. (1985), Ranch *et al*. (1985), Barwale *et al*. (1986), Ghazi *et al*. (1986), Lippmann and Lippmann (1993), Santarem and Finer (1999), Aragao *et al*. (2000), Yan *et al*. (2000), Walker and Parrot (2001) and Ko *et al*. (2004). These researchers were able to produce somatic embryogenesis from different genotypes using callus cultures derived from immature embryos that were able to develop into whole plants. An embryogenic, regenerable suspension culture has also been described by Finer (1988), Finer and Nagasawa (1988) and Sato *et al*. (1993). More recently, Sairam *et al*. (2003) reported on the regeneration of soybean plants from cotyledonary nodal callus.

Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. One of the disadvantages of soybean embryogenic cultures is that a series of different media are used to accommodate the different stages of embryogenesis, making the regeneration process time and labour consuming (Bailey *et al*., 1994; Trick *et al*., 1997). Also, sterility and chromosomal

abberations of primary transformants have been associated with age of the embryogenic suspensions (Singh *et al*., 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue.

Soybean plant regeneration has also been possible through organogenesis, using epicotyls (Wright *et al*., 1987b) and apical meristems (Kartha *et al*., 1981; Barwale *et al*., 1986; McCabe *et al*., 1988; Sato *et al*., 1993). Plant regeneration from cotyledonary nodes (Cheng *et al*., 1980; Wright *et al*., 1986; Kaneda *et al*., 1997; Zhang *et al*., 1999a; Olhoft and Somers, 2001; Shan *et al*., 2005), hypocotyls (Kaneda *et al*., 1997; Dan and Reichert, 1998; Reichert *et al*., 2003) and cotyledons (Hinchee *et al*., 1988) and primary leaf tissue (Wright *et al*., 1987a) has also resulted in reliable methods of regeneration of fertile soybean plants. Multiple shoots were obtained from genotypes tested and resulted in the generation of complete plants (Kaneda *et al*., 1997). No morphological differences were noted among regenerants, or between them and seed initiated plants in the study done by Reichert *et al*. (2003). All regenerants produced viable seed that germinated and produced morphological normal plants (Reichert *et al*., 2003).

Several groups have reported plant regeneration from protoplasts isolated from various tissues, namely roots, leaves (Wei and Xu, 1988), immature embryos and cotyledons (Myers *et al*., 1989; Dhir *et al*., 1992; Widholm *et al*., 1992) of both

wild and cultivated soybean. Recently, Rodrigues *et al*. (2005) reported on the embryogenic responses in soybean anther culture.

For legumes that have been regarded as recalcitrant to transformation, regeneration *in vitro* is highly genotype specific and only rarely are cultivated varieties amenable to regeneration. In these cases, regeneration remains more of an 'art' than a science (Grant *et al*., 2003). In addition, regeneration is often slow and the frequency of transformation (number of transformed plants generated from each explant) is often low (Somers *et al*., 2003). Expanding the range of genotypes within a species that undergo the requisite tissue culture process would provide a major contribution to improving the transformation system (Somers *et al*., 2003).

The shoot apical meristem presents an excellent explant for plant regeneration, particularly in species for which tissue culture regeneration is not well established and thus possess considerable potential for genotype independent gene transfer to recalcitrant species (Potrykus, 1990). Efficient and reproducible plant regeneration systems producing fertile plants have been developed from shoot apices of aseptically germinated seedlings of sorghum (Zhong *et al*., 1998), castor (Sujatha and Reddy, 1998), pearl millet (Devi *et al*., 2000), cotton (Gould *et al*., 1991a; McCabe and Martinell, 1993; Saeed *et al*., 1997; Hemphill *et al*., 1998; Zapata *et al*., 1999a and b; Satyavathi *et al*., 2002) and pear (Matsuda *et al*. 2005).

Cytokinins in general induce shoot organogenesis in cultured tissues (Thorpe, 1993) and there have been many reports on the effect of various cytokinins on shoot organogenesis in grain legumes (Polisetty *et al*., 1997; Sreenivasu *et al*., 1998; Tivarekar and Eapen, 2001; Shan *et al*., 2005). The effects of the cytokinin 6-benzyladenine (BA) on shoot organogenesis from the shoot apical meristem of soybean has been reported (Kartha *et al*., 1981; Barwale *et al*., 1986, Sato *et al*., 1993). However, there have been no reports on the effects of the cytokinins kinetin, zeatin, and thidiazuron (*N*-phenyl-*N*'-1,2,3-thiadiazol-5-ylurea; TDZ) on shoot organogenesis from the shoot apical meristem of soybean. TDZ is a substituted phenylurea compound with cytokinin-like activity (Mok *et al*., 1982, Visser *et al*., 1992). TDZ is useful for rapid plant regeneration of several species through organogenesis (Malik and Saxena, 1992a and b; Sharma *et al*., 2004) or somatic embryogenesis (Kim *et al*., 1997; Sreenivasu *et al*., 1998). It can stimulate shoot multiplication either alone or in combination with other growth regulators. TDZ has been found to be less susceptible to plant's degrading enzymes than endogenous cytokinins. It is active at lower concentrations than the amino purine cytokinins (Mok *et al*., 1997). Moreover, plant regeneration can be stimulated through exposure to TDZ for a relatively short time (Visser, 1992). TDZ has been used to regenerate multiple bud tissues from cotyledonary nodes of soybean (Shan *et al*., 2005). TDZ has been reported to facilitate multiple shoot proliferation from cotyledonary nodes and hypocotyls of soybean (Kaneda *et al*., 1997), a shoot apex culture of kenaf (*Hibiscus cannabinus* L.) (Srivatanakul *et al*., 2000) and orchid shoot tip sections (Malabadi *et al*., 2004). The shoot organogenesis of common bean, peanut and pigeonpea, crops known to be

relatively recalcitrant in tissue culture, was achieved using TDZ (Malik and Saxena, 1992a; Kanyand *et al*., 1994; Sreenivasu *et al*., 1998; Eapen *et al*., 1998).

In South Africa, soybean is cultivated successfully in Mpumalanga, KwaZulu-Natal, Free State, Northern Province, Gauteng and North West Province (http://www.nda.agric.za). The importance of this crop in South Africa is illustrated by the fact that the area planted with this legume increases annually (http://www.nda.agric.za). In addition to animal feed requirements, demands for a source of cheap protein by our expanding population have resulted in a sharp increase in South African soybean production over the past few years. Soybean competes favourably with maize on an economic basis (Duxbury *et al*., 1990) and there is considerable potential for expanding soybean production in South Africa. Recent developments in gene transfer techniques provide an opportunity to modify commercially grown South African soybean cultivars by producing transgenic plants. To establish a working system for genetically engineering South African grown varieties, efficient regeneration protocols are necessary.

In view of the potentially economic importance of soybean for small-scale and commercial farmers in South Africa, and the potential to improve commercially grown cultivars by genetic transformation, an efficient regeneration protocol utilizing the shoot apical meristem was developed from South African soybean cultivars, Talana and Ibis. Reported in this chapter are the results of the experiments and the different effects of kinetin, BA, zeatin and TDZ on the percentage of shoot apical meristems producing multiple shoots. To our

knowledge, this is the first report on the regeneration of commercially grown South African soybean cultivars Talana and Ibis from the shoot apical meristem.

3.2 Materials and methods

3.2.1 Explant preparation

Seeds of commercially grown South African soybean cultivars, Talana and Ibis, were surface sterilized by washing briefly in soapy water and then soaking in 2.6% (v/v) sodium hypochlorite solution containing three drops of Triton X-100 per 200 ml for 12 min. Following this step and under sterile conditions, the seeds were placed in 70% (v/v) ethanol for 1.5 min. The seeds were then thoroughly rinsed four times in sterile distilled water (Bryne *et al*., 1987). Sterilized seeds were left overnight in sterile distilled water at room temperature and then placed in sterile petri dishes containing 20 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Appendix A), pH 5.8. The seeds were germinated under growth chamber conditions (25±2°C; 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 $\mu Em^{-2} s^{-1}$) for 12-14 days. After 12-14 days, shoot apical meristems measuring 0.4-1 mm in length were aseptically isolated from the *in vitro* grown soybean seedlings under the dissecting microscope.

3.2.2 Induction of multiple shoots from shoot apical meristems

This experiment was designed to compare the cytokinins kinetin, zeatin, BA and TDZ with respect to their effects on the production of shoots from the shoot apical

meristems of Talana and Ibis. Shoot apical meristems isolated from 12-14 day old seedlings were placed onto 20 ml MS medium containing 1ml/l of 1000X MS vitamin stock solution (Sigma) (standard vitamin complement of MS), 2% (w/v) sucrose (BDH), 0.7% (w/v) purified agar (Oxoid), pH 5.8. Fifty explants were used for each concentration tested and 10 explants were placed in a petri dish (5 petri dishes/concentration tested). The cytokinins at the following concentrations: 0.0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 mg/l were added individually to the MS medium after autoclaving. Cultures were maintained at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μEm^{-2} s^{-1} and subcultured onto the same fresh medium bi-weekly. After four weeks of culture, the number of meristem explants forming multiple shoots were counted. The formation of more than two shoots by an explant was considered as induction of multiple shoots (Agrawal *et al*., 1997). This experiment was replicated twice. For each experiment, 50 explants were used for each concentration of cytokinin tested. The averages of shoot apical meristems producing multiple shoots was calculated and expressed as a percentage.

3.2.3 Statistical analysis

Standard errors from the means were calculated (Clarke and Cooke, 1989). Due to the fact that the dependent variable (the presence or absence of multiple shoots) was dichotomous and explanatory variables were present, a logistic regression model was used. The logistic regression was performed (SAS Enterprise Guide 3.0) to determine whether the cultivar, cytokinins kinetin, BAP, zeatin and TDZ

and varying concentrations of these cytokinins had a significant effect on the production of multiple shoots from Talana and Ibis shoot apical meristems. A logistic regression regresses the natural logarithm of the odds ratio on the explanatory variables. A logistic regression models the dependent variable, whether or not multiple shoots are present, on the explanatory variables, concentration, cytokinin and cultivar using the count data (frequency of shoot apical meristems producing shoots) corresponding to the different combinations of these variables. If Pr>ChiSq [probability associated with the test statistic (Wald Chi-Square) assuming no effect] <0.05, the effect is considered significant.

3.2.4 Rooting of *in vitro* grown shoots

Three hundred Talana and Ibis shoots, 2-3 cm in length, were excised from the shoot apical meristems cultured on 0.05 mg/l BA or 1 mg/l TDZ (the concentrations which gave the highest percentage of meristems producing multiple shoots) and placed onto media with and without various auxins to induce rooting. The following media were assessed: half-strength MS medium (Appendix A), pH 5.8, without any hormones, half-strength MS medium to which NAA or indole-3-butyric acid (IBA) at 0.05 mg/l and 0.1 mg/l were added and Gamborg's B5 medium (Gamborg and Wetter, 1975) (B5) (Appendix A) without hormones. Fifty Talana and Ibis shoots were placed onto each media. Cultures were incubated at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 $\mu Em^{-2} s^{-1}$.

3.2.5 Shoot regeneration on MS medium containing BA and NAA

MS medium containing both a cytokinin (BA) and an auxin (NAA) was assessed for its potential in initiating and maintaining shoot formation from shoot apical meristems. One hundred shoot apical meristems of Talana and Ibis, measuring 0.4-1 mm in length were aseptically isolated from 10-12 day old seedlings. These shoot apical meristems were cultured in petri dishes filled with 20 ml MS medium containing 0.05 mg/l BA and 0.2 mg/l NAA, pH 5.8 (MS soybean regeneration medium) (Appendix E). All explants were maintained at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 $\mu Em^{-2}\ s^{-1}$. Regenerated shoots, 2-3 cm tall, were excised from the shoot meristems and placed onto B5 medium (Appendix A) without hormones for rooting to occur. Complete plantlets (5-6 cm) were transferred to 30 ml sterile potting mixture (Appendix C) in sealed tissue culture flasks (10 cm x 5 cm diameter) for further growth. Plantlets were maintained in the growth chamber at 25±2°C with a 16:8 h photoperiod with light supplied at an intensity of 35-40 $\mu Em^{-2}\ s^{-1}$. After a month in tissue culture flasks, plantlets were transferred to sterile potting mixture (as above) in pots (12 cm x 14.5 cm diameter). Potted plantlets were placed into the growth chamber (at 25±2°C with a 16:8 h photoperiod with light supplied at an intensity of 35-40 $\mu Em^{-2}\ s^{-1}$) and were acclimatized by enclosure within glass jars to generate a humid environment. After 2-3 weeks, the glass jars were removed. Regenerated plants were watered daily and a nutritional solution of Multifeed Classic (Plaaskem) (Appendix C) was used once a week. For plant development, the regenerated plants were maintained at 25±2°C under growth lights with a 16:8

h light:dark photoperiod with light supplied at an intensity of 35-40 μEm^{-2} s^{-1}. Visual observations (height of plant; shape, width and length of leaves; size, shape and colour of flowers) of regenerated plants were compared to seed-initiated plants.

3.3 Results

3.3.1 Shoot regeneration on medium containing various cytokinins

The effects of BA, TDZ, kinetin and zeatin on shoot organogenesis from the shoot apical meristems of Talana and Ibis were studied. Multiple shoot production from Talana and Ibis shoot apical meristems was noticed within 3-4 weeks of culture initiation. In the present study, we observed that the different cytokinins and concentrations of these cytokinins had a significant effect (Table 3.1) on the production of multiple shoots from the shoot apical meristems of Talana and Ibis (Figs. 3.1 and 3.2).

Two or more shoots were directly induced from the shoot apical meristems placed on MS medium. The percentages of shoot apical meristems producing shoots ranged from 18-21% for Talana and 16-20% for Ibis (Figs. 3.1 and 3.2).

Multiple shoots (3-7 per meristem) were directly induced from the shoot apical meristems of both Talana and Ibis by BA and TDZ at all concentrations (Figs. 3.1 and 3.2).

Table 3.1: Analysis of effects from the logistic regression for Talana and Ibis.

Effect	DF	Wald Chi-Square	Pr>ChiSq
Concentration	1	30.1236	<0.0001
Cytokinin	3	129.5999	<0.0001
Cultivar	1	2.0081	0.1565
Concentration*Cultivar	1	0.0549	0.8147
Concentration*Cytokinin	3	53.5430	<0.0001
Cultivar*Cytokinin	3	0.6475	0.8855

If Pr>ChiSq <0.05, the effect is considered significant

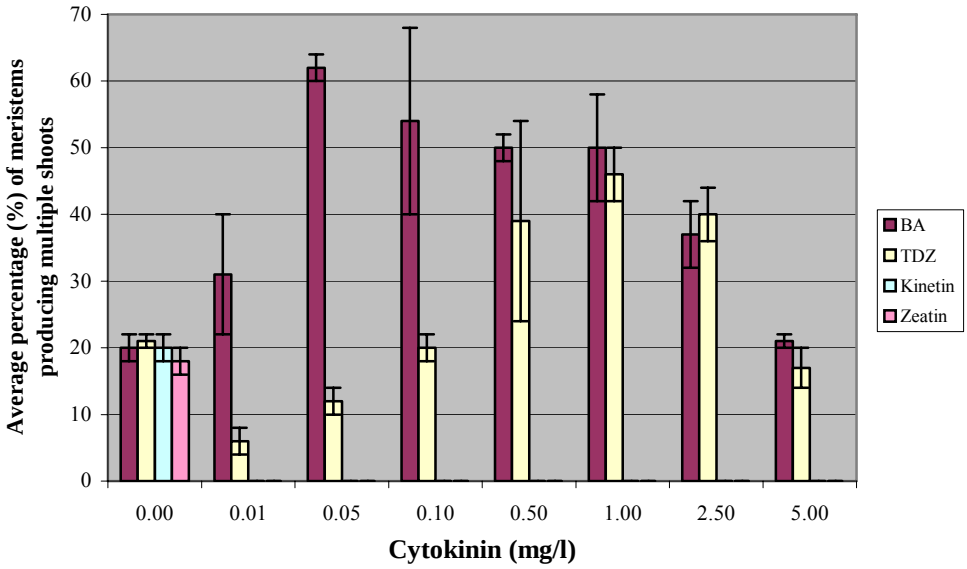

Figure 3.1: Average percentage of Talana shoot apical meristems producing multiple shoots at different concentrations of cytokinins BA, TDZ, kinetin and zeatin. Values are the means of two replicates with 50 explants in each replication. Error bars represent the standard error from the mean.

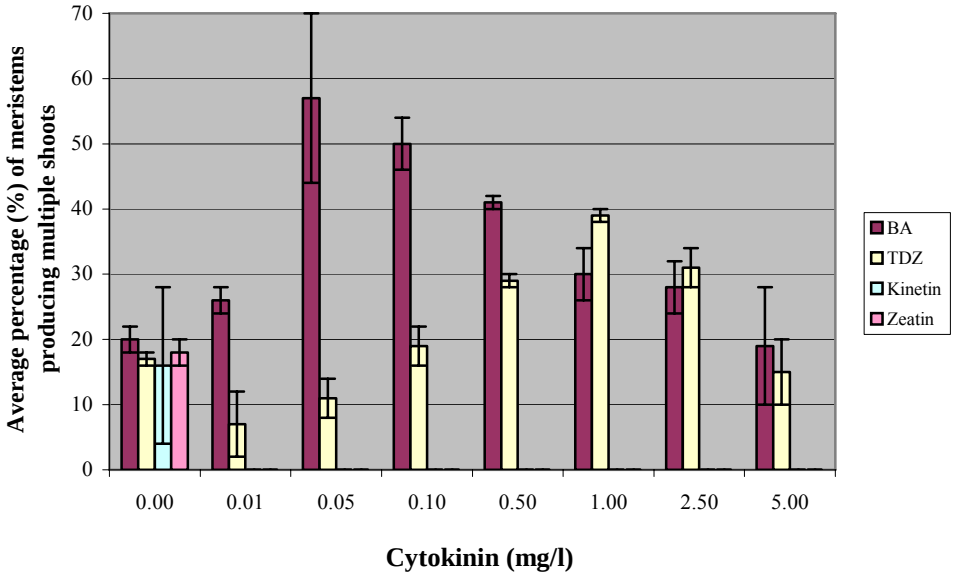

Figure 3.2: Average percentage of Ibis shoot apical meristems producing multiple shoots at different concentrations of cytokinins BA, TDZ, kinetin and zeatin. Values are the means of two replicates with 50 explants in each replication. Error bars represent the standard error from the mean.

Kinetin and zeatin did not induce multiple shoot formation from the shoot apical meristems of Talana and Ibis at any of the concentrations (Figs. 3.1 and 3.2). Media containing these cytokinins caused loss of apex organization and the formation of green callus. The callus exhibited vitrification (Srivatanakul *et al*., 2000) as characterized by a water-soaked appearance and translucent tissue.

The percentage of shoot apical meristems of Talana producing multiple shoots was influenced by both type and concentration of cytokinin. The highest percentage (62%) of shoot meristems of Talana producing multiple shoots was obtained on regeneration medium containing 0.05 mg/l BA (Fig. 3.1). Six to seven shoots per meristem were produced. The percentages (21-54%) of meristems with multiple shoots were reduced at lower (0.01 mg/l) and higher (0.1-5.0 mg/l) BA concentrations (Fig. 3.1). When placed on regeneration medium containing TDZ, the highest percentage (46%) of Talana shoot apical meristems producing multiple shoots was obtained on regeneration medium containing 1 mg/l TDZ (Fig. 3.1). Six to seven shoots per meristem were produced. The percentages (6-40%) of meristems producing multiple shoots were reduced at lower (0.01-0.5 mg/l) and higher (2.5 and 5.0 mg/l) TDZ concentrations (Fig. 3.1).

The highest percentage (57%) of Ibis shoot meristems with multiple shoots was obtained on regeneration medium containing 0.05 mg/l BA (Fig. 3.2). Six to seven shoots per meristem were produced. The percentages (19-50%) of explants producing multiple shoots were reduced at lower (0.01 mg/l) and higher (0.1-5.0

mg/l) BA concentrations (Fig. 3.2). When placed on regeneration medium containing TDZ, the highest percentage (39%) of shoot meristems of Ibis with multiple shoots was obtained on regeneration medium containing 1 mg/l TDZ (Fig. 3.2). Five to six shoots per meristem were produced. The percentages (7-31%) of explants producing multiple shoots were reduced at lower (0.01-0.5 mg/l) and higher (2.5 and 5.0 mg/l) TDZ concentrations (Fig. 3.2).

Low concentrations (0.01 and 0.05 mg/l) of TDZ were detrimental to the percentages (6% for Talana and 7% for Ibis) of shoot meristems producing multiple shoots when compared with the percentages (21% for Talana and 17% for Ibis) of shoot meristems producing multiple shoots on medium without TDZ.

At the concentration of 0.05 mg/l BA, the percentage (62%) of shoot apical meristems of Talana producing multiple shoots was not considerably higher than the percentage (57%) of shoot apical meristems of Ibis with multiple shoots. Similarly, at the concentration of 1 mg/l TDZ, the percentage (46%) of shoot apical meristems of Talana producing multiple shoots was not considerably higher than the percentage (39%) of shoot apical meristems of Ibis with multiple shoots. The type of cultivar did not have a significant effect (Table 3.1) on the production of multiple shoots from the shoot apical meristems at the concentrations of the different cytokinins tested.

Three hundred regenerated shoots (2-3 cm) from Talana and Ibis shoot meristems, which were excised and placed onto half-strength MS medium without any

hormones, half-strength MS medium with NAA or IBA at 0.05 mg/l and 0.1 mg/l and Gamborg's B5 medium, did not produce roots and became necrotic within a week. All shoots regenerated on cytokinins alone could not be rooted.

3.3.2 Shoot regeneration on MS medium containing BA and NAA

Talana and Ibis seeds were aseptically germinated after which shoot apical meristems were excised and placed onto soybean regeneration medium (MS medium containing 0.05 mg/l BA and 0.2 mg/l NAA) for shoot regeneration. The earliest sign of growth from 100% of Talana and Ibis meristems was noticeable within one week of culture. Soybean meristems differentiated into multiple green shoots (6-7 per meristem for both Talana and Ibis) accompanied by callus as seen in Figs. 3.3 and 3.4. Three hundred excised Talana and Ibis shoots (2-3 cm), rooted on B5 medium without hormones. Shoots regenerated on cytokinins alone could not be rooted but if NAA was added to the medium, they could be properly rooted. Figure 3.5 shows three week old Talana plantlets on B5 medium. After four weeks, shoots had elongated, more shoots proliferated and a well developed rooting system had appeared. The overall percentage of rooted shoots from the isolated shoots was 66% (198/300) for Talana and 52% (156/300) for Ibis. Complete plantlets (5-6 cm) with many shoots and an established rooting system were transferred to sterile potting mixture in tissue culture flasks. After a month 100% Talana and Ibis plantlets developed new shoots that green and healthy (Fig. 3.6). All regenerated plants were transferred to sterile potting mixture in pots and acclimatized successfully in the growth chamber.

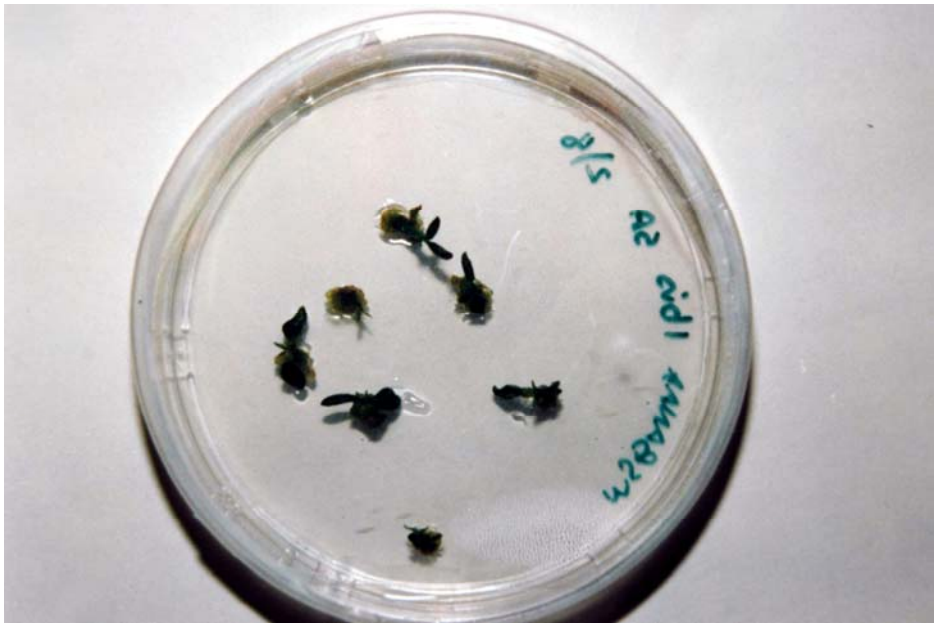

Figure 3.3: Callus (c) and shoot (s) formation within one week from shoot apical meristems of Ibis on medium containing 0.05 mg/l BA and 0.2 mg/l NAA (*bar* 10 mm).

Figure 3.4: Green callus (c) and shoots (s) formed within one week from shoot apical meristems of Talana on medium containing 0.05 mg/l BA and 0.2 mg/l NAA (*bar* 10 mm).

Figure 3.5: Three week old Talana plantlets on B5 medium (*bar* 10 mm).

Figure 3.6: Regenerated Talana plantlet with new shoots and a good rooting system transferred to potting mixture for further growth (*bar* 10 mm).

These regenerated plants were phenotypically (height of plant; shape, width and length of leaves; size, shape and colour of flowers normal) normal when compared to seed-initiated plants, and set seed (Fig. 3.7). Normal, fertile plants were therefore regenerated from Talana and Ibis shoot apical meristems. The overall frequency of regenerated soybean plants per meristem was 66% for Talana and 52% for Ibis.

3.4 Discussion

A major limitation to the genetic transformation of soybean has been the establishment of an efficient procedure for the regeneration of plants from cultured tissues. Although the production of callus and somatic embryos has been widely reported for soybean (Lazzeri *et al*., 1985; Ranch *et al*., 1985; Barwale *et al*., 1986; Lippmann and Lippmann, 1993; Santarem and Finer, 1999; Aragao *et al*., 2000; Yan *et al*., 2000 and Walker and Parrot 2001; Sairam *et al*., 2003; Ko *et al*., 2004), the regeneration of intact plants from such cultures requires a long time period and chromosomal abnormalities have been reported (Bailey *et al*., 1994; Trick *et al*., 1997; Singh *et al*., 1998). Regeneration of soybean plants from shoot meristems of cultivars Mandarin (Kartha *et al*., 1981) and Williams 82 (Sato *et al*., 1993) has been reported.

In this study, efforts were made to develop a rapid, simple, and reproducible regeneration protocol for commercially rown South African soybean cultivars, Talana and Ibis that can be used for transformation studies.

Figure 3.7: Regenerated Talana plants in potting mixture (*bar* 10 mm).

The effect of BA, kinetin, zeatin and TDZ, and the combination of BA and NAA, on shoot organogenesis from Talana and Ibis shoot apical meristems was assessed.

In this study, 16-20% of Ibis shoot apical meristems and 18-21% of Talana shoot apical meristems produced multiple shoots on MS medium. When compared with the highest percentages (62% for Talana and 57% for Ibis) of shoot apical meristems producing multiple shoots on medium containing 0.05 mg/l BA, for example, it was evident that cytokinins at a certain concentrations, are therefore necessary and important for high shoot apical meristem regeneration percentages.

The production of multiple shoots from the shoot apical meristems was influenced by the choice and concentration of cytokinin (Table 3.1). All concentrations of BA and TDZ produced multiple shoots from the shoot apical meristems of Talana and Ibis, the highest percentage of shoot apical meristems producing multiple shoots achieved at 0.05 mg/l BA and 1.0 mg/l TDZ for both cultivars. These two cytokinins were therefore concluded to be effective cytokinins at these concentrations for soybean shoot organogenesis from the shoot apical meristem.

Other researchers have reported on the efficiency of BA on the production of shoots from soybean explants other than shoot meristems. Kaneda *et al*. (1997) reported the highest percentage (6.7%) of multiple shoot induction from soybean (cultivar Bonminori) hypocotyl segments when 1.15 mg/l BA was added to half-strength L2 regeneration medium and the highest percentage (4%) of multiple

shoot induction from soybean (cultivar Bonminori) cotyledonary nodes when 1.15 mg/l BA was added to regeneration medium. Regeneration medium used in this study was MS, whereas half-strength LS medium was used in the study by Kaneda *et al*. (1997) because MS medium proved ineffective in promoting shoot organogenesis from the hypocotyls and cotyledonary nodes in their study. Therefore, the type of medium used for regeneration also has an effect on the regeneration frequencies. It has been reported that basal medium influences soybean regeneration frequencies (Wright *et al*., 1987a and b; Shetty *et al*., 1992). Sato *et al*. (1993) and McCabe *et al*. (1988) used MS medium for the regeneration of shoots from the shoot apical meristems of soybean. Sato *et al*. (1993) reported on regenerated shoots from shoot apical meristems of the soybean cultivar Williams 82 on MS medium containing 3 mg/l BA and 0.037 mg/l NAA.

BA is, therefore, an excellent cytokinin for shoot organogenesis from the shoot apical meristems of soybean. Sujatha and Reddy (1998) reported that BA at a concentration of 2 mg/l was found to be superior to the other cytokinins (kinetin, TDZ, BA) for obtaining the highest number of shoots (46.7) from the shoot apex of castor. Zhong *et al*. (1998) also reported that 2 or 4 mg/l BA added to MS medium initiated adventitious buds from the shoot apices of sorghum. In contrast, other researchers (Zapata *et al*., 1999b) have reported the failure of BA to stimulate multiple shoots from the shoot apex of kenaf. High concentrations of BA resulted in callus formation from the shoot apical meristem of kenaf (Zapata *et al*., 1999b).

This is the first report on the effect of TDZ on shoot regeneration from shoot apical meristems of soybean. When comparing the highest percentages of shoot apical meristems of Talana and Ibis producing multiple shoots on medium containing 0.05 mg/l BA (62% and 57%, respectively) and 1.0 mg/l TDZ (46% and 39%, respectively), it was evident that TDZ is not as efficient as BA in inducing shoot organogenesis from the shoot apical meristems. It was also evident that low concentrations (e.g. 0.01 mg/l) of TDZ were detrimental to the percentages (6% for Talana and 7% for Ibis) of meristem explants producing multiple shoots when compared to the percentages (21% for Talana and 17% for Ibis) of meristem explants producing multiple shoots on medium without TDZ.

The best concentrations of TDZ (1.0 and 2.5 mg/l) for shoot regeneration from Talana and Ibis shoot meristems are similar to those reported by Kaneda *et al*. (1997). In their study, they found that 1-2 mg/l TDZ was the best concentration for soybean (cultivar Bonminori) shoot organogenesis from hypocotyls. The optimal TDZ concentration in peanut tissue culture was also reported to be 2 mg/l (Saxena *et al*., 1992). In contrast, Shan *et al*. (2005) reported that the highest initiation of multiple bud tissues at the cotyledonary nodes of soybean (cultivar White hilum) was achieved when 0.1 mg/l TDZ was added to the medium. The range of optimal TDZ concentrations for the soybean tended to be narrower than that for *Phaseolus vulgaris* (Malik and Saxena, 1992a) and the peanut (Kanyand *et al*., 1994).

The best concentrations of TDZ for shoot regeneration vary among species and varieties (Saxena *et al*., 1992; Kanyand *et al*., 1994; Kim *et al*., 1997; Sujatha *et al*., 1998). Multiple shoot initiation by 1 µmol/l TDZ from shoot apices of kenaf has been reported (Srivatanakul *et al*., 2000). Eapen *et al*. (1998) reported that TDZ alone (1 or 2 mg/l) or in combination with (0.1 mg/l) IAA induced high frequency shoot regeneration from primary leaf segments of three pigeonpea cultivars. In contrast, Satyavathi *et al*. (2002) reported that TDZ (0.05- 0.3 mg/l) resulted in the formation of callus from the cut ends of cotton shoot tips and delay in shoot response.

Both Talana and Ibis shoot apical meristems responded uniformly on medium containing varying concentrations of BA and TDZ. Although variations in the percentages of shoot apical meristems producing multiple shoots were recorded with each different concentration and cytokinin, both cultivars responded more or less similarly. The type of cultivar did not have a significant effect (Table 3.1) on shoot production from the shoot apical meristems of Talana and Ibis. Therefore, the shoot regeneration system described in this study is possibly genotype independent at particular concentrations. Similarly, Sairam *et al*. (2003) reported an efficient genotype independent callus-mediated regeneration protocol from soybean (cultivars Williams 82, Loda and Newton) using cotyledonary nodal callus. In their study, when different hormones were used, the frequency of both callus and regeneration was likewise modified. Although variations in the frequency of callus induction and regeneration were observed with each different plant growth regulator combination, all of the tested genotypes responded more or

less similarly (Sairam *et al*., 2003). Zhong *et al*. (1998) reported on a genotype independent regeneration protocol from shoot apices of sorghum. Although the highest regeneration frequency in the genotypes tested varied from 65% to 99% depending on the culture medium and genotypes, all 18 genotypes tested responded to multiplication of shoot apices at a relatively high efficiency on different combinations of 2,4-D and BA. Satyavathi *et al*. (2002) also reported that genotype seemed to have not much effect on shoot induction and shoot proliferation from the shoot apical meristems of three cotton cultivars. This was evident from the similar response of all varieties on different hormonal concentrations studied. Gould *et al*. (1991a) also reported genotype independent regeneration from shoot apex tissues of cotton.

The cytokinins kinetin and zeatin did not induce shoots from the shoot apical meristems of Talana and Ibis. This is the first report on the effect of kinetin and zeatin on shoot regeneration from shoot apical meristems of soybean. These results are, however, in keeping with other researchers (Tivarekar and Eapen, 2001) who have reported that kinetin and zeatin failed to induce shoots from a different explant source, i.e. immature cotyledons of mungbean. It can therefore be concluded that kinetin and zeatin (at the concentrations tested) are not effective cytokinins for soybean organogenesis from the shoot apical meristems of Talana and Ibis.

Cytokinins are known to induce axillary as well as adventitious shoot formation from meristematic explants (Sujatha and Reddy, 1998). TDZ has exhibited a

strong cytokinin activity in several cultures. TDZ has been reported to induce multiple shoot formation in various dicotyledons (Eapen *et al*., 1998). It has been successfully used in many tissue culture protocols for different grain leguminous plants known to be somewhat recalcitrant to regeneration procedures such as pea, chickpea and lentil (Malik and Saxena, 1992b), common bean (Malik and Saxena, 1992a; Cruz de Carvalho *et al*., 2000) and soybean (Kaneda *et al*., 1997). It has been shown to promote shoot regeneration at a much lower concentration than other cytokinins, and shoots regenerated with comparable or greater efficiency than with other cytokinins (Lu, 1993; Kaneda *et al*., 1997; Srivatanakul *et al*., 2000). The mechanisms of action of TDZ are not completely understood. TDZ may be involved in the preprogramming and expression of competent cells necessary for them to undergo differentiation and development. The different potential modes of action of TDZ have been reviewed by Murthy *et al*. (1998). TDZ was found to induce synthesis or accumulation of endogenous cytokinins (Thomas and Katterman, 1986; Hutchinson and Saxena, 1996). This may result from an increase in synthesis, a decrease in catabolism, or a conversion of storage forms to biologically active cytokinins. Murch *et al*. (1997) suggested TDZ induced stress in plants in culture. The formation of regenerated plants may be an adaptive reproduction mechanism of plants to overcome the stress induced by TDZ.

The effectiveness of tissue-culture regimes is dependent on many factors including plant species, tissue type, size of explant, chemical properties and concentration of cytokinins and auxins (Bowen, 1993).

In this study, MS medium was suitable for shoot organogenesis from the shoot apical meristems of Talana and Ibis. These results contrast the results of studies done by other researchers (Wright *et al*., 1986; Shetty *et al*., 1992; Kaneda *et al*., 1997). These researchers reported that although MS or modified MS was often used for soybean shoot organogenesis, it did not seem to be suitable for organogenesis since it had minimal effects on shoot formation. The high salt concentrations in the MS formulation influence shoot organogenesis (Kaneda *et al*., 1997).

All shoots from the shoot apical meristems of Talana and Ibis which were placed onto all rooting media containing auxins did not produce roots and became necrotic within a week and so no regenerated plantlets were obtained. These results are similar to reports by other researchers (Huettenman and Preece, 1993; Lu, 1993) that one of the disadvantages of using TDZ in culture medium is the difficulty in rooting of regenerated shoots. Soybean might not contain high auxin levels that are favourable for rooting and which do not overcome a 'carry over' effect of TDZ from the multiple shoot induction medium (Srivatanakul *et al*., 2000). However, these results contrast the results by other researchers who reported that regenerated soybean (cultivar Bonminori) shoots from hypocotyl segments cultured on TDZ, elongated and rooted when transferred to medium containing 0.1 mg/l IBA (Kaneda *et al*., 1997). Other researchers have reported that rooting of cotton meristems is also problematic and may be genotype-dependent (Gould *et al*., 1991a; Hemphill *et al*., 1998). Grafting of meristems to

seedling rootstocks offers a means to circumvent the rooting problem (Luo and Gould, 1999).

In this study, the effect of both cytokinin BA and auxin NAA (added to the regeneration medium) on the percentage of shoot apical meristems producing multiple shoots and the percentage of regenerated plants from these meristem explants was assessed. This experiment was conducted because no regenerated plants were obtained from shoot apical meristems of both cultivars placed on medium containing only cytokinins for shoot induction. On the medium containing BA and NAA, both cultivars produced multiple shoots (6-7 per meristem) from 100% of the meristems. These shoots then rooted on B5 medium to obtain a high frequency of regenerated plantlets per meristem (66% for Talana and 52% for Ibis). Shoots regenerated on cyokinins alone could not be rooted (3.3.1) but if NAA was added to the regeneration medium they could be properly rooted. The regenerated Talana and Ibis plantlets in this study were successfully transferred to potting mixture for further growth and development. The regeneration experiment has demonstrated a rapid, reproducible, efficient and successful technique with a high rate of regeneration of soybean plants from tissue culture utilizing shoot apical meristem-derived explant material. The regenerated plants from cultivars Ibis and Talana which were produced within four weeks were phenotypically (height of plant; shape, width and length of leaves; size, shape and colour of flowers) normal, produced normal flowers and set seed. This is the first report of plant regeneration from shoot apical meristems of these South

African cultivars. This regeneration system has the potential for use in experiments to genetically transform South African soybean cultivars.

Whole plant development from soybean shoot meristems has been reported by Kartha *et al*. (1981). The soybean cultivar Mandarin was tested and meristems cultured on medium containing BA and NAA at concentrations of 0.1 and 1 μM respectively (optimum level for whole plant regeneration) regenerated into whole plants at a frequency of 33%. Shoots and rooting occurred from the shoot meristem. This plant regeneration protocol would not be suitable for transformation studies because of possible chimeric shoots being produced from the shoot apex during transformation and therefore resulting in a transformed plant with transformed and non-transformed shoots. In this study, Talana and Ibis shoots that regenerated from the shoot apical meristem on medium containing BA and NAA were excised and then placed onto B5 rooting medium (devoid of plant growth regulators) for plantlet formation. The higher frequencies (66% for Talana and 52% for Ibis) of regenerated plants in this study compared to 33% obtained by Kartha *et al*. (1981) is possibly due to the cultivar types. In contrast with plant regeneration via the induction of embryogenic callus in soybean tissue cultures reported earlier (Lazzeri *et al*., 1985; Ranch *et al*., 1985; Barwale *et al*., 1986; Ghazi *et al*., 1986; Lippmann and Lippmann, 1993), the regeneration protocol described in this study appears to be less genotype-dependent.

In previous regeneration trials in this study with the individual use of BA in the culture medium, meristems differentiated into multiple shoots (3.3.1). Root

regeneration on rooting medium and subsequent plantlet formation did not occur. It thus appeared that a critical concentration of BA and NAA is needed from the beginning of the regeneration protocol for regeneration of soybean using shoot apical meristems to obtain regenerated plants with a good rooting system. Similarly, Sato *et al*. (1993) reported that shoot tips of the soybean cultivar Williams 82 placed onto MS medium containing 3 mg/l BA and 0.037 mg/l NAA, produced shoots, which when excised from the original explant and placed onto B5 medium with no hormones, produced roots and plantlets were formed. Satyavathi *et al*. (2002) reported that the highest multiple shoot regeneration occurred from the shoot tip of cotton cultivars cultivated on MS medium containing 0.1mg/l BA and 0.1 mg/l NAA, which, when excised from the explant, rooted on half strength MS medium containing 0.3 mg/l IBA.

The combination of both a cytokinin and auxin in the regeneration medium is essential for good rooting frequencies (66% for Talana and 52% for Ibis) of regenerated shoots as seen in this study. Kartha *et al*. (1981) reported that other legumes (cowpea, chickpea, and peanut) required various concentrations of BA and NAA for plant regeneration from shoot apices. They reported that extremely low levels of BA (0.1-0.005 μM) in association with low levels of NAA (0.05 μM) induced cowpea plant regeneration at a high frequency (100%). When 0.1 μM BA and 10 μM NAA was added to regeneration medium, whole peanut plantlets could be regenerated at a frequency of 75%. Recently, Tivarekar and Eapen (2001) reported that on media supplemented with a combination of a cytokinin and auxin, immature cotyledons of mungbean isolated from embryos

produced high frequency shoot regeneration. The combination of 2 mg/l BA and 0.5 mg/l IAA produced the highest frequency of shoot regeneration. When these regenerated shoots were excised and cultured on half strength MS medium supplemented with IBA (0.1 mg/l), rooting occurred and complete plants were obtained (Tivarekar and Eapen, 2001). Eapen *et al*. (1998) reported that TDZ alone (1 or 2 mg/l) or in combination with (0.1 mg/l) IAA induced high frequency shoot regeneration from primary leaf segments of three pigeonpea cultivars and that rooting of 90 % of the shoots was obtained on MS medium containing 1 mg/l NAA. Zhong *et al* (1998) reported that when shoot apices of sorghum were placed onto medium containing 2 or 4 mg/l BA and 0.5 mg/l 2,4-D, adventitious shoots were formed, which then rooted in medium containing 1 mg/l IBA.

Histological observations by Sato *et al*. (1993) of several stages of regenerating shoot tips have indicated that shoot organogenesis in soybean apical meristems involves multiple cells. The entire meristematic region of both axillary and primary shoot apices appears to be involved in *de novo* shoot organogenesis (Sato *et al*., 1993). This indicated that the regenerated soybean shoots are likely to be of multicellular origin, and that several layers of cells are involved in shoot organogenesis.

Adventitious soybean shoots have been induced from epicotyls (Wright *et al*., 1987b), cotyledonary nodes (Cheng *et al*., 1980; Wright *et al*., 1986; Kaneda *et al*., 1997; Zhang *et al*., 1999a; Olhoft and Somers, 2001; Shan *et al*., 2005), hypocotyls (Kaneda *et al*., 1997; Dan and Reichert, 1998; Reichert *et al*., 2003),

cotyledons (Hinchee *et al*., 1988) and primary leaf tissue (Wright *et al*., 1987a). Shoot meristems of soybean seedlings have seldom been used as explants (Kartha *et al*., 1981; Barwale *et al*., 1996; Sato *et al*., 1993). On the basis of the results obtained in this study, the shoot apical meristem seems to be a remarkable tissue culture explant for soybean and can be used for regeneration studies.

The advantages of shoot meristem culture over other regeneration systems are many fold. Shoot regeneration from shoot apical meristem is direct, relatively simple and needs less time to regenerate large numbers of plants (Saeed *et al*., 1997). Plants regenerated from shoot meristems are true to phenotype with low incidence of somaclonal variation and chromosomal abnormalities (Bajaj, 1998). The shoot meristem explant has few genotype limitations and is considered as more appropriate because meristematic cells are programmed for direct shoot organogenesis without an intervening callus stage (Zapata *et al*., 1999b). However, the disadvantage is that direct organogenesis from a meristem that has been subjected to *Agrobacterium* transformation or particle gun acceleration will will result in chimaeric plants (Sato *et al*.,1993).

The effect of combinations of cytokinins BA, TDZ, kinetin and zeatin used in this study on the regeneration of shoots from the shoot apical meristems could also be assessed in future studies, as well as combinations with various auxins to improve rooting and plant regeneration frequencies. To determine if this regeneration method is genotype independent, other commercially grown South African soybean cultivars need to be evaluated in future studies.

3.5 Conclusions

The type of cytokinin and the concentration of the cytokinin had a significant effect on the production of shoots from the shoot apical meristems of Talana and Ibis. The type of cultivar did not have a significant effect on the production of shoots from the shoot apical meristem explants. The best concentration of BA for shoot organogenesis from the shoot apical meristems of the soybean cultivars, Talana and Ibis, is 0.05 mg/l. The best concentration of TDZ for shoot organogenesis from the shoot apical meristems of Talana and Ibis is 1 mg/l. As both cultivars responded uniformly on medium containing varying concentrations of BA and TDZ, this regeneration system is possibly genotype independent. Kinetin and zeatin did not induce shoots from the soybean shoot apical meristems and are therefore not effective cytokinins for shoot organogenesis. All regenerated shoots from shoot apical meristems cultured on BA or TDZ alone did not form roots on rooting media used in this study.

The establishment of an efficient and reproducible plant regeneration system from the shoot apical meristems of two commercially grown soybean cultivars, Talana and Ibis, is described in this study. The overall frequency of regenerated soybean plants per meristem was 66% for Talana and 52% for Ibis. This is the first report of a successful regeneration protocol for the South African soybean cultivars, Talana and Ibis. The procedure developed is rapid, simple, possibly genotype independent and involves the use of both a cytokinin (BA) and auxin (NAA) in the regeneration medium. The success of the current work on the regeneration of

commercially grown South African soybean cultivars from shoot apical meristems and the high frequency of plant regeneration indicates that soybean plant tissue culture now can be used in regeneration technology for crop improvement, through gene transfer e.g. the *pat* gene for herbicide resistance, in soybean.

CHAPTER 4

Transformation of the shoot apical meristems of commercially grown South African soybean cultivars with *Agrobacterium tumefaciens* containing the *pat* gene for resistance to the herbicide phosphinothricin.

Sections of this chapter have been presented as a paper at the Biotechnology for Africa 95-All African Conference on Biotechnology, University of Pretoria, South Africa, 13-15 November 1995.

C.R. Viljoen, C. McNaughton and V.H. Whitlock. Genetic manipulations and transfer of the phosphinothricin acetyltransferase (*pat*) gene into tobacco and soybean.

Abstract

In search of establishing a system for genetic transformation of South African commercially grown soybean (*Glycine max* cvs Talana and Ibis) for improving cultivars, a method of *Agrobacterium*-mediated transformation of isolated shoot apical meristems of seedlings was developed to produce herbicide resistant plants. Prior to transformation experiments, soybean cultivars, Talana and Ibis, were screened for susceptibility to virulent *A. tumefaciens* sis 43. Both cultivars produced tumours (85% for Talana and 70% for Ibis) in response to infection and were therefore compatible hosts for *Agrobacterium*-mediated DNA transfer. Prior to transformation experiments the concentrations of the antibiotics, cefotaxime and carbenicillin, to effectively eliminate *Agrobacterium* but not affect shoot regeneration, were determined. These were found to be 250 mg/l and 10 mg/l for cefotaxime and carbenicillin, respectively. The concentrations of the selection agents PPT and kanamycin in the selection medium, which would kill non-transformed shoot apical meristems and shoots, were also determined. Concentrations of 1, 5 and 20 mg/l PPT and 100, 200 and 250 mg/l kanamycin, inhibited shoot and root formation for both cultivars. The concentrations of 1 mg/l PPT and 100 mg/l kanamycin were therefore chosen for selection of transformants. The effect of wounding (with a needle) and injecting water and 20 μM acetosyringone on shoot regeneration from 20 shoot apical meristems isolated from 12 day old Talana and Ibis seedlings was assessed prior to transformation experiments. After four weeks in culture, 100% multiple shoot regeneration occurred from these wounded meristems indicating no damage to meristem tissue.

One hundred shoot apical meristems (0.4-1 mm in length) isolated from 12 day old Talana and Ibis seedlings were wounded three times with a sterile needle and then transformed by submerging for 30-40 min in the 48 h *A. tumefaciens* strain LBA4404 (harbouring the recombinant vector pBI101 containing the *pat* gene and the marker gene *npt II*). After the transformed shoot apical meristems were co-cultivated with *Agrobacterium* for three days on SH medium containing 20 μM acetosyringone and 10 mM glucose, they were transferred to regeneration medium containing 250 mg/l cefotaxime and 10 mg/l carbenicillin. After a month they were transferred to regeneration medium containing 1 mg/l PPT and 100 mg/l kanamycin for selection of transformed shoots. One hundred percent non-transformed control explants turned brown and became necrotic within one week on the selection medium. After two months on selection medium, two Talana shoot meristems out of 100 produced healthy shoots (15 shoots from both meristems) indicating that the shoot apical meristems were transformed with the *pat* gene that was successfully detoxifying the herbicide PPT. One hundred percent shoot meristems of Ibis did not produce transformed shoots and became necrotic within two weeks. Only eight shoots (3-5 cm) from the initial 15 putatively transformed Talana shoot meristems produced roots on B5 medium containing 100 mg/l kanamycin and 1 mg/l PPT. The percentage of rooted Talana shoots was therefore 53%. After one month eight healthy plantlets were formed. The overall transformation frequency from the initial 100 Talana shoot apical meristems to transformed plants in tissue culture was 1.06%. The presence of the *pat* gene in shoots from the transformed soybean plants was confirmed by PCR, indicating successful transformation of meristematic tissues and the presence of

non-chimeric transgenic plants. The eight transgenic plants with healthy roots were transferred to sterile potting mixture in tissue culture flasks and then acclimatized in the growth chamber. All these transformed plantlets lost their leaves and died two weeks later.

4.1 Introduction

Soybean (*Glycine max* L. Merrill) is the world's most important oil seed and grain legume and has been a prominent target for improvement by molecular techniques of genetic modification (Christou *et al*., 1997; Somers *et al*, 2003). These molecular techniques allow the incorporation or modification of genetic traits that cannot be introduced or modified by classical plant-breeding methods. However, progress in transgenic improvement in soybean is constrained by extant transformation systems and inefficient regeneration systems (Olhoft *et al*., 2003). A soybean transformation system must have a number of attributes for it to be useful in both basic research and cultivar improvement through genetic engineering. An ideal transformation method for soybean should: (i) be simple, inexpensive and rapid; (ii) provide efficient selection without production of non-transformed or chimeric plants; and (iii) be successful with a range of cultivars and, if an *Agrobacterium*-mediated method, *A. tumefaciens* strains and binary plasmids (Olhoft *et al*., 2003).

The development of gene transfer techniques for soybean is of commercial interest as they will facilitate the production of cultivars with improved

characteristics such as resistance to environmental stress and disease, enhanced nutritional value, as well as tolerance to specific herbicides. The transfer and expression of foreign genes in plant cells will also facilitate the study of gene expression.

Foreign DNA has been incorporated into soybean genome resulting in transformed plants. Introduction of agronomically important traits into soybean has been accomplished via plant transformation by *Agrobacterium*-mediated transfer systems (Hinchee *et al*., 1988; Parrot *et al*., 1989; Di *et al*., 1996; Zhang *et al*., 1999a; Clemente *et al*., 2000; Yan *et al*., 2000; Ke *et al*., 2001; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Ko *et al*., 2004; Zeng *et al*., 2004; Shan *et al*., 2005), direct infection of germinating seeds (non-tissue-culture approach) with *Agrobacterium* (Chee *et al*., 1989; de Rhonde *et al*., 2001) and with DNA-coated microprojectiles (McCabe *et al*., 1988; Christou *et al*., 1989; Finer *et al*., 1992; Sato *et al*., 1993; Moore *et al*., 1994; Parrot *et al*., 1994; Hadi *et al*., 1996; Stewart *et al*., 1996; Ponappa *et al*., 1999; Santarem and Finer, 1999; Aragao *et al*., 2000; Srinivasa Reddy *et al*., 2003). Transgenic plants have been obtained with each technique and the foreign genes inserted into these plants were stably inherited in the progeny of the transformed plants (McCabe *et al*., 1988; Clemente *et al*., 2000; Yan *et al*., 2000; Olhoft *et al*., 2003; Zeng *et al*., 2004). Agronomic important traits include herbicide tolerance (Padgette *et al*., 1995; Zhang *et al*., 1999a; Clemente *et al*., 2000; Olhoft and Somers, 2001), amino acid modification (Falco *et al*., 1995), viral resistance (Di *et al*., 1996) and insect resistance (Parrot *et al*., 1994; Stewart *et al*., 1996). However, soybean transformation methods are

not routinely reproducible and routine recovery of transgenic soybean plants has been restricted to a few genotypes (Christou, 1997), with no reports on the transformation of commercially grown South African soybean cultivars Talana and Ibis. Plants recovered were also often chimeric (Parrot *et al*., 1989; Meurer *et al*., 1998). Recently, transformation efficiency of soybean has been increased from 0.4-3% (Hinchee *et al*., 1988; Di *et al*., 1996; Zhang et al., 1999a; Donaldson and Simmonds, 2000) to 16.4% (Olhoft *et al*., 2003).

Agrobacterium-mediated DNA transformation has some advantages over direct transformation methods in reducing the copy number of the transgene and potentially leading to fewer problems with transgene co-suppression and instability. Other advantages include defined transgene integration and fewer rearrangements within the inserts (Kohli *et al*., 1999). The use of this transformation system normally results in clonally transformed plants, in contrast to the large number of mosaic plants usually obtained by direct transformation (Neuhuber *et al*., 1994; Bastar *et al*., 2004). One significant limitation of this method, however, is that transformation appears to be genotype dependent.

Agrobacterium-mediated transformation of soybean has been reported using cotyledons (Hinchee *et al*., 1988; Clemente *et al*., 2000), immature cotyledons (Parrot *et al*., 1989; Santarem *et al*., 1998; Yan *et al*., 2000), cotyledonary nodes (Di *et al*., 1996; Meurer *et al*., 1998; Zhang *et al*., 1999a; Donaldson and Simmonds, 2000; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Zeng *et al*., 2004; Shan *et al*., 2005), embryogenic suspension cultures (Trick and Finer, 1998) and

somatic embryos (Ko *et al*., 2004). Ke *et al*. (2001) reported on the *Agrobacterium*-mediated transformation of the three sections of germinated soybean seedlings, namely, intact cotyledon plus a segment of the hypocotyl; part of a cotyledon plus a segment of the hypocotyl; and the adjacent hypocotyl.

The cotyledonary node method is a frequently used soybean transformation system based on *Agrobacterium*-mediated T-DNA delivery into regenerable cells in the axillary meristems of the cotyledonary node (Di *et al*., 1996; Meurer *et al*., 1998; Zhang *et al*., 1999a; Donaldson and Simmonds, 2000; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Zeng *et al*., 2004; Shan *et al*., 2005). Improvements have been reported in cotyledonary node transformation systems (Palanichelvam *et al*., 2000; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Zeng *et al*., 2004), however, while these studies represent substantial progress towards improving the cotyledonary node system, the production of transgenic soybean plants remains inefficient. Efforts to overcome problems with host/tissue specificity of *Agrobacterium* and low (0.4-3%) transformation efficiency of soybean include the use of hypervirluent/modified *Agrobacterium* strains, adding thiol compounds to the co-cultivation medium, or sonicating target tissues prior to co-cultivation (Hood *et al*., 1993; Trick and Finer, 1997; Santarem *et al*., 1998; Ke *et al*., 2001; Olhoft and Somers, 2001; Olhoft *et al*., 2003; Zeng *et al*., 2004). In addition to the problems of host-explant specificity, the cotyledonary node is multicellular in origin and so regenerated shoots will have a multicellular origin. Thus, chimerism in regenerated transgenic soybean plants is a problem (Yan *et al*., 2000).

Regeneration of Talana and Ibis soybean plants from shoot apical meristems has been developed successfully (Chapter 3). These explants can therefore be used for transformation studies using *Agrobacterium* or particle bombardment. Using the shoot apical meristem regeneration system, transgenic progeny may be directly produced by transformation of cells in the LII layer. The primary transformants will always be chimeric (Lowe *et al*., 1995; Zhong *et al*., 1996). The relatively stability of chimeric shoots depends on the original position of the transformed cell(s) and the competition between transformed and non-transformed cells (Marcotrigiano, 1990). Alternatively, manipulation of the transgene meristem cells by growth regulator treatments that induce multiple shoot regeneration from the shoot meristem could produce more stable transformants.

Transformation of shoot apical meristems by *Agrobacterium* has been reported for corn, (Gould *et al*., 1991b), banana (May *et al*., 1995), rice (Park *et al*., 1996), sunflower (Alibert *et al*., 1999), lupin (Li *et al*., 2000), pear (Matsuda *et al*., 2005) and cotton (Zapata *et al*., 1999a; Satyavathi *et al*., 2002). Normal fertile plants have been produced (Gould *et al*., 1991b; Park *et al*., 1996; Li *et al*., 2000). Leaves from different areas of transformants were assayed because of the possibility that the plants were chimeric for the transferred traits (Gould *et al*., 1991b). To our knowledge, there have been no reports on the transformation of shoot apical meristems of the soybean cultivars Talana and Ibis by *Agrobacterium*-mediated DNA transfer.

Particle bombardment has been the method of choice for obtaining transgenic soybean (McCabe *et al*., 1988; Christou *et al*., 1989; Finer and McMullen, 1991; Sato *et al*., 1993; Parrot *et al*., 1994; Ponappa *et al*., 1999; Srinivasa Reddy *et al*., 2003), as many of the genotypes are recalcitrant to *Agrobacterium*-mediated transformation. However, particle bombardment very often results in multiple transgene integration events, and the presence of multiple copies of a transgene has been shown to result in transgene silencing in many instances (Srinivasa Reddy *et al*., 2003). Transformation of soybean shoot apical meristems by particle bombardment has been reported by McCabe *et al*. (1988) and Sato *et al*. (1993). Chimeric, transgenic shoot primordia and plants were produced.

The use of herbicides to control weeds allows growers to practice more efficient crop management, together with improving yields. Several classes of herbicides can be effective for broad-spectrum weed control, but these are either non-selective, killing also the crop plants, or they significantly injure some crops at the application rates required (Fox, 1990). Engineering herbicide tolerance into crops represents a new alternative for conferring selectivity of herbicides and enhancing crop safety. The production of herbicide resistant commercially grown South African soybean plants to a single, highly efficient non-selective herbicide without toxicity to animals and rapid degradation in the soil would be welcomed, as these transgenic plants would provide more effective, less costly and more environmentally attractive weed control.

Two general approaches have been taken in engineering herbicide resistance or tolerance in plants. The first approach involves altering the level and sensitivity of the target enzyme for the herbicide and the second approach involves incorporating a gene that will detoxify the herbicide before it reaches the biochemical target inside the plant cell (Stalker et al., 1988; Gasser and Fraley, 1992).

The herbicide resistance genes *bar* (Thompson *et al*., 1987) and *pat* (Bayer *et al*. 1972), have been widely used in transformation experiments (D' Halluin *et al*., 1992). The *pat* gene, isolated from *Streptomyces viridochromogenes* Tu494 (Bayer *et al*., 1972; Wohlleben *et al*., 1988; Alijah *et al*., 1991) encodes for the enzyme phosphinothricin acetyltransferase (PAT), which acetylates the free ammonium group of phosphinothricin (PPT), the active ingredient of the herbicide Basta, thereby rendering the latter non-toxic (Murakami *et al*., 1986; De Block *et al*., 1987; Thompson *et al*., 1987; Strauch *et al*., 1988). Transgenic PPT resistant plants expressing the *pat* or *bar* gene include soybean (Zeng *et al*., 2004), cassava (Sarria *et al*., 2000), oat and barley (Zhang *et al*., 1999b; Bregitzer and Tonks, 2003), sugar cane (Leibbrandt and Snyman, 2003), perennial grass (Shu *et al*., 2005) and carrots (Chen and Punja, 2002).

In recent years, a major effort has been made in several laboratories to engineer herbicide resistant plants. This research has yielded promising results in the case of resistance to glyphosate (Comai *et al*., 1985; Shah *et al*., 1986; Mazur and Falco, 1989; Padgette *et al*., 1995; Penaloza-Vazquez *et al*., 1995; Schmidt, 1995;

Zhou *et al*., 1995; Clemente *et al*., 2000), bromoxynil (Stalker *et al*., 1988), the sulfonylurea and imidazolinone herbicides (de Greef *et al*., 1989) and phosphinothricin and glufosinate (De Block *et al*., 1987; D'Halluin *et al*., 1992; Zhou *et al*., 1995; Park *et al*., 1996; McCabe *et al*., 1999; Zhang *et al*., 1999a; Sarria *et al*., 2000; Chen and Punja, 2002; Choi *et al*., 2003; Goldman *et al*., 2003; Leibbrandt and Snyman, 2003; Manickavasagam *et al*., 2004; Zeng *et al*., 2004; Shu *et al*., 2005). The development of herbicide-resistant crop plants was one of the first commercial applications of plant genetic engineering (Freyssinet and Cole, 1999). Commercial application of herbicide resistance in the field has been reported for tobacco and potato (de Greef *et al*., 1989), sugarbeet (Buckman *et al*., 2000), rice (Jiang *et al*., 2000), soybean (Somers *et al*., 2003) and cotton (May *et al*., 2004).

Soybean is cultivated successfully in many parts of South Africa. Soybeans are cultivated under dryland conditions and grown mainly in Mpumalanga and KwaZulu-Natal, while small quantities are also cultivated in the Free State, Northern Province, Gauteng and North West Province (http://www.nda.agric.za). The importance of this crop in South Africa is illustrated by the fact that the area planted with this legume increases annually (http://www.nda.agric.za). In addition to animal feed requirements, demands for a cheap source of protein by our expanding population have resulted in a sharp increase in South African soybean production over the past few years. Soybean competes favourably with maize on an economic basis (Duxbury *et al*., 1990) and there is considerable potential for expanding soybean production in South Africa.

Genetically modified herbicide (glyphosate) resistant soybean has been approved for commercial use in South Africa. Recent developments in gene transfer techniques provide an opportunity to modify commercially grown South African soybean cultivars to produce plants that are resistant to the herbicide Basta.

In view of the economic importance of soybean in South Africa and the potential to improve commercially grown cultivars by genetic transformation, a transformation protocol, utilising the shoot apical meristem and *Agrobacterium*-mediated DNA transfer, was developed for the South African soybean cultivar Talana.

4.2 Materials and methods

4.2.1 *Agrobacterium* susceptibility test

4.2.1.1 Bacterial strains

Descriptions and maintenance of the bacterial strains used in the susceptibility study are given below. Stock cultures were maintained as streaks at 4^{o}C and subcultured onto fresh medium every two weeks.

- *A. tumefaciens* LBA4404 is a non-oncogenic octopine strain and harbours the resident disarmed pAL4404. This bacterium was maintained at 30^{o}C on LB medium (Appendix B) containing 100 mg/l rifampicin (Sigma).

- Virulent *Agrobacterium* sis 43 was obtained from Dr. J. Burger (Agricultural Research Council, Roodeplaat, Pretoria) and was maintained at 30^{o}C on yeast-

mannitol-congo red (YMCR) medium (Appendix B). This strain harbours a hypervirulence plasmid and is oncogenic.

For the susceptibility test, *A. tumefaciens* sis 43 was used to inoculate the soybean cultivars, Talana and Ibis and the sunflower plants. This virulent *Agrobacterium* strain was grown on YMCR medium at 30^{o}C for 48 h. The avirulent *A. tumefaciens* strain LBA4404 (negative control) also used for inoculation of soybean cultivars was grown on LB rifampicin (100 mg/l) agar plates at 30^{o}C for 48 h.

4.2.1.2 Growth and inoculation conditions for the *Agrobacterium* susceptibility test

Growth and inoculation conditions were according to Byrne *et al*. (1987) and Owens and Cress (1985), with modifications. Six Talana and Ibis seeds and six sunflower seeds (positive control for comparative purposes) were planted in potting mixture (Appendix C) in 12 cm x 15 cm diameter pots. In the growth chamber, cool white fluorescent lighting was provided on a 12:12 h light: dark photoperiod and the temperature was 25±2°C. Light was supplied at an intensity of 35-40 μE m^{-2} s^{-1}. Humidity was not controlled. Plants were watered daily. Twenty five days after sowing, plants were inoculated by pinprick puncture. Uniform wounding was achieved by use of a wounding tool consisting of a small, flat piece of wood through which five pins were arranged along a 1 cm line. The pin tips, protruding about 1 mm through the wood, were gently pressed into the

stem to the maximum extent possible (Owens and Cress, 1985). The wounding tool was sterilized by autoclaving for 40 min at 121°C before use.

A bacterial colony was scraped from the 48 h bacterial plate with a sterile toothpick and placed onto the sterile pins. The plant was then wounded as described above. Inoculations were made along the entire length of the stem at intervals of 1 cm. Ten inoculations were made per plant and wounded sites were covered with petroleum jelly to prevent bacterial desiccation (Byrne *et al*., 1987). The soybean plants were inoculated as follows: four plants with the virulent *Agrobacterium* sis 43, one plant with the avirulent *Agrobacterium* LBA4404 and one plant was wounded with the wounding tool containing no bacteria. The sunflower plants were inoculated as follows: one plant was wounded with the wounding tool containing no bacteria and five plants were inoculated with the virulent *Agrobacterium* sis 43. Five weeks after inoculation, soybean and sunflower plants were scored visually for tumour formation. The total number of tumours per plant of each cultivar was recorded. Percent tumour formation was calculated as follows (Byrne *et al*., 1987):

$$\text{Percent tumour formation} = \frac{\text{number of tumours on four plants}}{\text{number of inoculation sites (10/plant)}} \times 100$$

4.2.1.3 Growth and inoculation of aseptically grown plants (*in vitro* culture of plants) for the *Agrobacterium* susceptibility test

Six soybean seeds (Talana and Ibis) were surface sterilized by washing briefly in soapy water and then soaking in 2.6% (v/v) sodium hypochlorite solution containing three drops of Triton X-100 per 200 ml for 12 min. Following this step and under sterile conditions, the seeds were placed in 70% (v/v) ethanol for 1.5 min. The seeds were then thoroughly rinsed four times in sterile distilled water (Byrne *et al*., 1987). Sterilized seeds were left overnight in sterile distilled water at room temperature and placed onto one-half strength Schenk and Hildebrandt (Schenk and Hildebrandt, 1972) (SH) medium (Appendix A) in large test tubes sealed with cotton wool stoppers and placed at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}. Sunflower (*Helianthus annuus* Linn.) seeds were surface sterilized by washing briefly in soapy water, rinsed and then soaked for 20 min in 2.6% (v/v) sodium hypochlorite containing three drops of Triton X per 200 ml. The seeds were then transferred to 70% (v/v) ethanol for 5 min and then rinsed four times in sterile distilled water (M^{c}Kenzie and Cress, 1992). The sunflower seeds were then placed onto one-half strength SH medium (Appendix A) in large test tubes sealed with cotton wool stoppers and placed at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}.

Fourteen to eighteen days after sowing, soybean and sunflower plants were cut aseptically at the cotyledonary node and a 48 h virulent or avirulent bacterial

colony was applied to the cut surface with a sterile toothpick. Optimal tumour formation required the removal of axillary shoots emerging from the cotyledonary node as well (Byrne *et al*., 1987). Plants were incubated at 25±2°C with a 16:8 h photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1} and tumour formation scored after five weeks. Two sunflower plants were inoculated with the virulent culture, one Talana and Ibis plant with the virulent culture, one Talana and Ibis plant with the avirulent culture and one plant of each cultivar was cut and no bacteria added to the cut surface.

4.2.1.4 Culturing of tumours

After one month, tumours from the plants grown in the growth chamber were excised, washed in soapy water for 5 min, sterilized by soaking in 2.6% (v/v) sodium hypochlorite for 10 min, followed by 70% (v/v) ethanol for 1 min, then rinsed four times in sterile distilled water. The external part of the tumour was excised and discarded and the internal part cut into approximately 3 mm cubes and placed on hormone-free PC-L2 medium (Phillips and Collins, 1979) (Appendix E) containing 500 mg/l carbenicillin (Sigma) (Byrne *et al*., 1987). Tumours were subcultured every four to six weeks. Tumours incited on aseptically grown plants were transferred directly onto hormone-free PC-L2 medium containing carbenicillin without surface sterilization. Fresh tumours on greenhouse and *in vitro* grown plants were excised, weighed and the diameter measured (Owens and Cress, 1985).

4.2.2 Transformation of shoot apical meristems

4.2.2.1 Explant preparation

Seeds of commercially-grown South African soybean cultivars Talana and Ibis, were surface sterilized as previously described (4.2.1.3). Sterilized seeds were left overnight in sterile distilled water at room temperature and then placed in sterilized petri dishes containing 20 ml of MS medium (Appendix A), pH 5.8. The seeds were germinated under growth chamber conditions (25±2°C; 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}) for 12-14 days. After 12-14 days, shoot apical meristems measuring 0.4-1 mm in length were aseptically isolated from the *in vitro* grown soybean seedlings.

4.2.2.2 Determination of the concentrations of antibacterial and selection agents for use in transformation experiments

Prior to transformation trials, the following were determined for soybean shoot apical meristems:

1) The concentrations of the antibacterial agents, namely carbenicillin and cefotaxime, which inhibited growth of *Agrobacterium*, but still allowed shoot regeneration from the shoot apical meristems and root regeneration from the elongated shoots. Talana and Ibis shoot apical meristems were placed onto soybean shoot regeneration medium (Appendix E) containing 250 mg/l cefotaxime (Sigma) and 10 mg/l carbenicillin (Al-Janabi and Shoemaker,

1992). Shoots (3-5 cm tall) were excised and placed onto B5 medium (Appendix A) containing 250 mg/l cefotaxime and 10 mg/l carbenicillin, for root induction. Soybean explants were also placed onto the above medium containing no antibacterial agents for comparative purposes.

2) The minimum concentrations of the selection agents, namely kanamycin (Boehringer Mannheim) and PPT, which inhibited shoot regeneration and rooting. The following media were used:

   Soybean shoot regeneration medium and B5 medium containing: no selective agents; 1 mg/l PPT and 100 mg/l kanamycin; 5 mg/l PPT and 200 mg/l kanamycin; 20 mg/l PPT and 250 mg/l kanamycin.

Soybean seeds (Talana and Ibis) were sterilized as described previously (4.2.2.1) and placed on MS medium for germination. After 12-14 days, seedlings were removed from the germination medium and placed into sterile petri dishes. Shoot apical meristems measuring 0.4-1 mm in length were aseptically isolated from the *in vitro* grown soybean seedlings. Ten shoot apical meristems from Talana and Ibis seedlings were placed on the selective medium above for shoot induction. The petri dishes were sealed with parafilm and the explants incubated with a 16:8 h light: dark photoperiod with light supplied at an intensity of 35-40 $\mu E\ m^{-2}\ s^{-1}$. The explants were observed regularly for the formation of shoots. Shoots (3-5 cm tall), which were induced from the shoot apical meristems on non-selective medium, were excised and placed onto B5 medium with selection agents, for root induction. The experiments were repeated three times for reproducibility of

results. Average regeneration frequency was calculated and expressed as a percentage.

4.2.2.3 The effect on the production of shoots by injecting shoot apical meristems with sterile distilled water and acetosyringone

Twenty shoot apical meristems of each cultivar (Talana and Ibis) were isolated from 12 day old seedlings (4.2.2.1), wounded three times with a hypodermic needle and injected with 10 µl of sterile distilled water and 20 µM acetosyringone. The needle used was the following size: 27 *g* x ½ inch. Shoot apical meristems were then placed onto soybean regeneration medium (Appendix E) and the production of shoots was recorded over a period of three weeks.

4.2.2.4 Transformation of shoot apical meristems with *Agrobacterium*

Transconjugate *A. tumefaciens*, containing pBI101 with the *pat* and *npt* II selectable marker genes driven by the CaMV 35S promoter (Viljoen *et al*., 1995), was used for transformation experiments. This transformed *Agrobacterium* was maintained at 30^{o}C on LB medium (Appendix B) containing 100 mg/l rifampicin (Sigma) and 100 mg/l kanamycin (Boehringer Mannheim). A colony from a freshly streaked plate was inoculated into 20 ml of YEP medium (Appendix B) containing 10 mM glucose (BDH), 20 uM acetosyringone (Aldrich) (Khan *et al*., 1994), 100 mg/l rifampicin and 100 mg/l kanamycin. The isolate was grown to the exponential phase (OD_{600nm} = 0.6-0.8) at 30^{o}C with constant agitation at 160

rpm and then transferred to a centrifuge tube and centrifuged at 2 000 *g* for 15 min. The broth was decanted and the pellet resuspended in 18 ml YEP medium (at a density of OD_{600nm} = 0.6-0.8) containing 20 µM acetosyringone and 10 mM glucose (Khan *et al*., 1994).

Shoot apical meristems measuring 0.4-1 mm in length were first wounded three times with a hypodermic needle (27 *g* ½ inch needle attached to a 1 ml syringe containing the *Agrobacterium* culture) and at the same time inoculated with the *Agrobacterium*, and then submerged for 30-40 min in the transconjugate *Agrobacterium* culture. Excess culture was shaken off and the explants were placed onto SH medium (Appendix A) supplemented with 20 µM acetosyringone and 10 mM glucose (Khan *et al*., 1994) for three days co-cultivation with the *Agrobacterium* (Ke *et al*., 2001) at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. After three days co-cultivation, the meristems were placed onto soybean regeneration medium (Appendix E) containing 250 mg/l cefotaxime and 10 mg/l carbenicillin to kill the *Agrobacterium* and placed at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. Shoot apical meristems were transferred to fresh medium every three to four days to eliminate the *Agrobacterium*.

After a month, the putative transformed shoot apical meristems were transferred to soybean regeneration medium containing 1 mg/l PPT, 100 mg/l kanamycin, 250 mg/l cefotaxime and 10 mg/l carbenicillin for selection of transformed shoots.

Green, healthy shoots (3-5 cm tall) which continued to grow, were excised from the shoot apical meristem and transferred to soybean regeneration medium (Appendix E) containing 1 mg/l PPT, 100 mg/l kanamycin, 250 mg/l cefotaxime and 10 mg/l carbenicillin for selection of transformed shoots. Non-transformed control explants were placed onto selection medium as well as soybean regeneration medium without selection agents for comparative purposes. Transformed shoots were transferred to fresh medium every week to renew the medium and antibiotic and to exchange the culture atmosphere.

Transformation of meristems may result in chimeric plants. That is, only one cell in a cluster of meristematic cells might receive the foreign T-DNA, but the resulting enzymatic activity (transgene product) could protect the surrounding cells by kanamycin or PPT inactivation. As a result, a shoot could develop which would contain both transformed and non-transformed cells. As a first step in creating conditions to identify any possible non-transformed cells and cell-derivatives, a rooting regime in the presence of the selection agents, kanamycin and PPT, was used. Green, vigorously growing putative transgenic shoots cultured on soybean regeneration medium (Appendix E) with selection agents were placed onto B5 medium (Appendix A) containing selection agents (100 mg/l kanamycin and 1 mg/l PPT) to induce rooting. Potential transgenic plants with healthy roots were transferred to sterile potting mixture (Appendix C) in tissue culture flasks and placed into the growth chamber for further growth at 25±2^{o}C with a 16:8 h light: dark photoperiod with light supplied at an intensity of 35-40

$\mu E\ m^{-2}\ s^{-1}$. One hundred shoot apical meristem explants of Talana and Ibis were used for transformation.

To confirm the absence of the *Agrobacterium* around the transformed shoot apical meristems, an inoculation loop was placed several times around the putatively transformed shoot apical meristems (after the fourth or fifth subculture) and then the loop streaked onto LB plates containing the appropriate levels of antibiotics (as described in 4.2.1.1). LB plates inoculated with the transconjugate *Agrobacterium tumefaciens* served as the control. All plates were incubated at 25^{o}C for 48 h.

4.2.2.5 PCR analysis of transformed soybean shoots

To confirm integration of the *pat* gene in the genome of transformed shoots after transformation, PCR analysis was performed. Total DNA from leaves of one non-transformed soybean plant and new transformed shoots from each transformed soybean plant was isolated using the cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). PCR was performed using *pat* gene specific primers which amplified the 558 bp *pat* coding region of the 1.3 kb chimeric gene construct (including the promoter, coding region and terminator). The PCR Core Kit (Boehringer Mannheim) was used for each PCR reaction. The PCR reaction was carried out in a volume of 25 µl in a PCR tube.

Each reaction mixture contained 1X PCR reaction buffer [10 mM Tris-HCl, 50 mM KCL, pH 8.3 (20^{o}C)], 1.2 mM $MgCl_2$, 200 μM dNTP's (dATP, dCTP, dGTP, dTTP, pH 7.0), 0.5 μM of each of the 20 mer oligonucleotide primers (the sequences of the 20 mer oligonucleotide primers used were: left primer $^{5'}$ GTC TCC GGA GAG GAG ACC AG $^{3'}$ and right primer $^{5'}$ CCT AAC TGG CCT TGG AGG AG $^{3'}$) and 10 ng of template DNA. Sterile distilled water was added to give a total volume of 25 μl. The contents of the PCR tubes were mixed and centrifuged briefly at 13 000 *g*. The tubes were placed into the PCR machine and the template DNA denatured at 95^{o}C for 5 min. The tubes were place on ice, centrifuged briefly at 13 000 *g* and then 0.5 units of *Taq* DNA polymerase added. After mixing the contents of the tube and a brief centrifugation at 13 000 *g*, 25 μl of sterile mineral oil was added to the tube. The tubes were placed back into the PCR machine and the DNA amplified by 30 repetitive cycles involving template denaturation (92^{o}C for 1 min), primer annealing (60^{o}C for 1 min) and extension (72^{o}C for 1 min). The DNA was then extended for 5 min at 72^{o}C. The reaction was stopped by placing the tubes on ice. Samples subjected to PCR included DNA from a transformed soybean plant, DNA from a non-transformed soybean plant (negative control), water (negative control), pBI101/*pat* (positive control) and 1.3 kb chimeric gene construct (positive control). PCR products were separated on a 0.8% agarose gel stained with ethidium bromide to detect the amplified the *pat* 558 bp coding region. To demonstrate the efficient separation of the DNA fragment, the DNA molecular weight marker VI was used.

4.3 Results

4.3.1 Tumour formation on soybean and sunflower plants

The soybean cultivars, Talana and Ibis, were evaluated for susceptibility to *Agrobacterium* infection. Plants were scored visually for tumour formation five weeks after infection. Results of tumour formation on chamber-grown soybean and sunflower in response to infection with *A. tumefaciens* sis 43 are presented in Table 4.1.

Screening for *Agrobacterium* susceptibility at the whole plant level proved fruitful in identifying soybean cultivars, Talana and Ibis, capable of forming a compatible reaction with the pathogen. The data represented here confirms that Talana and Ibis are hosts for *A. tumefaciens* sis 43.

A. tumefaciens strain sis 43 incited tumours on the sunflower (Fig. 4.1) and the soybean cultivars Talana and Ibis (Figs. 4.2 and 4.3). A wide range of responses was observed among the soybean and sunflower plants. Ten days after inoculation, tumourous growth (>3 mm diameter) was apparent on the growth chamber-grown sunflower plants that had been inoculated with virulent *A. tumefaciens* sis 43. The tumours were large, ranging in mass from 0.006-1.045 g. Eighty eight percent of inoculations with *A. tumefaciens* sis 43 resulted in tumours (Table 4.1). On *in vitro* grown sunflower plants, tumours weighed an average of 0.35 g.

Table 4.1: Tumour formation on chamber-grown soybean and sunflower plants in response to infection with *A. tumefaciens* sis 43.

Cultivar	% tumour formation with *A. tumefaciens* sis 43	Tumour size	
		mm	g
Sunflower	88	>3	0.006-1.045
Talana	85	<3	0.004-0.020
Ibis	70	<3	0.003-0.092

Plants were scored five weeks after inoculation

Figure 4.1: *A. tumefaciens* sis 43 incited tumours (t) on the sunflower stem after five weeks (*bar* 10 mm).

Figure 4.2: *A. tumefaciens* sis 43 incited tumours (t) on the stem of the growth chamber-grown Talana plant after five weeks (*bar* 10 mm).

Figure 4.3: *A. tumefaciens* sis 43 incited tumours (t) on the stem of the growth chamber-grown Ibis plant after five weeks (*bar* 10 mm).

Figure 4.4: No tumours or callus developed on the control sunflower stem (s) which was wounded with the wounding tool (*bar* 10 mm).

No tumours or callus formation was evident on the wounded control sunflower plants (Fig. 4.4), or the sunflower plants inoculated with avirulent *Agrobacterium* LBA4404.

Talana and Ibis grown in the growth chamber were susceptible to virulent *Agrobacterium* strain sis 43 in terms of visual tumour formation evident within three weeks after inoculation, although tumours were small (<3 mm diameter) (Figs. 4.2 and 4.3). The tumours ranged in mass from 0.004-0.020 g for Talana and 0.003-0.092 g for Ibis (Table 4.1). Talana scored highest for tumour formation under growth chamber conditions, with tumours developing at 85 % of the inoculation sites. Tumours on Ibis developed at 70 % of the inoculation sites (Table 4.1). One to four small tumours were present at the inoculation sites. No tumours were formed on soybean plants inoculated with avirulent *Agrobacterium* LBA4404 (Fig. 4.5) or plants that were only wounded (Fig. 4.6). The tumours on the *in vitro* grown soybean weighed 0.0197 g and 0.0309 g for Ibis and Talana respectively, and were small (<2 mm) and clumped together.

One month after formation, tumours from Talana, Ibis and sunflower plants grown in the growth chamber and *in vitro* were successfully cultured on hormone-free medium. A month after tumours from the soybean cultivars were placed onto the PC-L2 medium, they grew rapidly and formed friable green-brown callus, and attained a size of 4-10 mm. Tumours from the sunflower plants formed hard green callus within ten days on the PC-L2 medium.

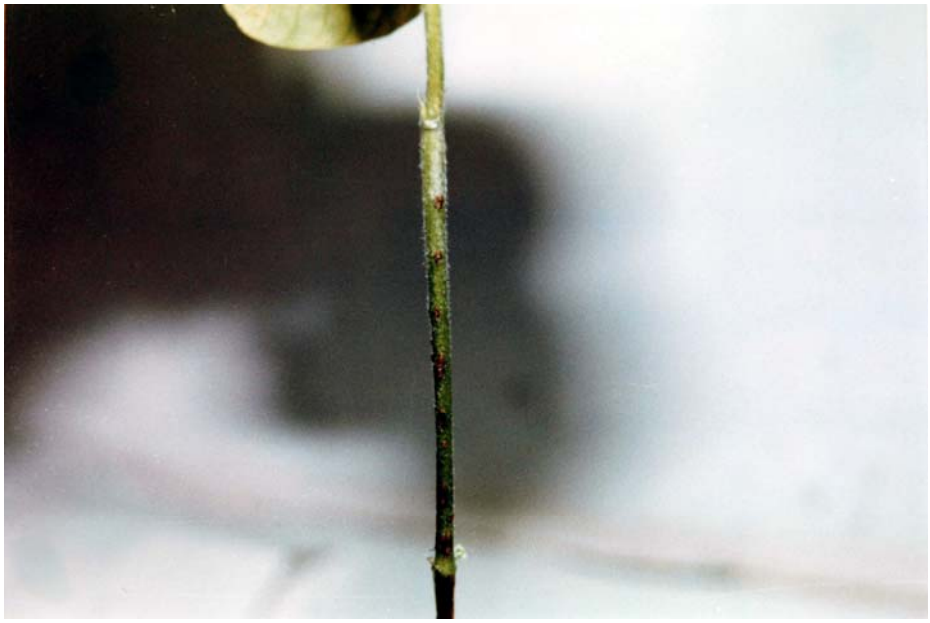

Figure 4.5: No tumours formed on the Talana stem inoculated with avirulent *Agrobacterium* LBA4404 (*bar* 10 mm).

Figure 4.6: No tumours or callus formed on the Talana stem following wounding with the wounding tool (*bar* 10 mm).

After two months tumours were still callusing on the medium. These tumours exhibited hormone-independent growth.

4.3.2 Explant preparation for transformation

Soybean seeds were surface decontaminated to achieve contamination free seedlings without adversely affecting the germination rate. The method used to surface sterilize soybean seeds was efficient since no or minimal contamination of seeds occurred and the sterilization method did not affect the germination of soybean seeds.

4.3.3 Determination of the concentrations of antibacterial and selection agents for use in transformation experiments

When soybean shoot apical meristems of Talana and Ibis were placed onto soybean regeneration medium containing 250 mg/l cefotaxime and 10 mg/l carbenicillin, 100% shoots were induced within a week from all meristems placed on the medium. All explants placed onto regeneration medium without antibacterial agents also formed shoots within a week. These concentrations of antibacterial agents, therefore, did not inhibit the regeneration of soybean.

All Talana and Ibis shoot apical meristems placed onto soybean regeneration medium containing varying concentrations of PPT and kanamycin did not produce any shoots and died within one week, compared to 100% explants on medium without selection agents which formed shoots that eventually rooted on B5

medium to form whole plants. All shoots induced from shoot apical meristems on medium without selection agents, which were placed on B5 medium containing selection agents, became necrotic and no rooting was observed. The concentrations tested can therefore be used in transformation studies to select for transformed explants and shoots.

4.3.4 Transformation of shoot apical meristems with *Agrobacterium*

Prior to transformation experiments, shoot production from the shoot apical meristems, after wounding and injecting water and acetosyringone into the meristems, was assessed. All meristems produced green healthy shoots within a week on soybean regeneration medium. After four weeks in culture, multiple shoots (6-7 per explant) were produced.

Talana and Ibis shoot apical meristems transformed with transconjugate *Agrobacterium* were allowed to grow for one month on soybean regeneration medium with antibiotics prior to transfer to medium containing 1 mg/l PPT and 100 mg/l kanamycin. Three days after culture on medium with antibiotics, all the transformed shoot apical meristems of Talana and Ibis gave rise to new shoots.

Transferring explants to fresh medium containing antibiotics every three to four days successfully eliminated the *Agrobacterium*. After the fourth subculture, no bacterial growth was observed on the test plates after 48 h of incubation, whereas normal bacterial growth was observed in controls. This result confirmed the

absence of *Agrobacterium* around the transformed soybean shoot apical meristems.

The emergence of resistant Talana shoot meristems producing shoots was observed after two months of continuous culture on selective medium containing PPT, with subculturing every two weeks. In contrast, the growth and differentiation of non-transformed explants on selective medium was completely inhibited. The shoot apical meristems showed signs of chlorosis and became necrotic one week later. The transformed Talana meristems on medium containing 1 mg/l PPT and 100 mg/l kanamycin continued to produce new shoots after two months, indicating that they contained the *pat* gene which was successfully detoxifying the herbicide PPT (Fig. 4.7). Only two transformed Talana shoot apical meristems, from the initial 100 explants transformed, survived after four months on the selection medium producing new shoots. Transformation efficiency of shoot meristems was therefore 2% for Talana (Table 4.2). No Ibis shoot apical meristems produced new shoots on selection medium and they became necrotic. Transformation efficiency of shoot meristems was therefore 0% for Ibis.

Fifteen putative transformed shoots (3-5 cm tall), from the multiplication of the two Talana meristems, were excised and placed onto B5 medium with selection agents, to induce rooting. Eight shoots produced roots and after one month eight healthy transformed plantlets were formed (Fig. 4.8). The percentage of rooted Talana shoots was therefore 53%. The overall transformation efficiency from the

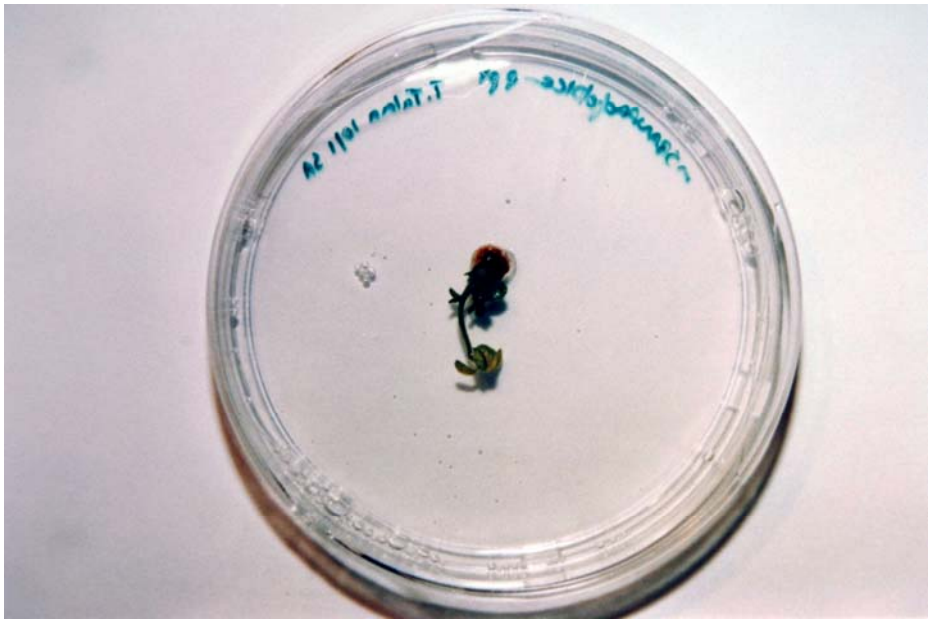

Figure 4.7: Transformed Talana shoot apical meristem on soybean regeneration medium containing 1 mg/l PPT and 100 mg/l kanamycin (*bar* 10 mm).

Figure 4.8: Transformed Talana plantlet on B5 medium (*bar* 10 mm).

Table 4.2: Results of *Agrobacterium* transformation of Talana shoot apical meristems.

% transformed meristems	No. transformed shoots	% rooted shoots	% transformation efficiency
2	15	53 (8/15)	1.06

initial 100 Talana explants to whole plants in tissue culture was 1.06%. The yield of regenerated plants was 8 from 100 initial meristems. The other seven shoots showed signs of chlorosis, did not produce roots and became necrotic within a week. When these transformed plantlets were transferred to soil for further development in the growth chamber, they lost their leaves and died two weeks later.

4.3.5 PCR analysis of transformed soybean shoots

To confirm the presence of the *pat* gene in the two transformed Talana plantlets, PCR was performed on DNA from various leaves of the transformed plantlets because of the possibility of chimeras. Figure 4.9 shows the results of PCR amplification for the *pat* gene from the genomic DNA. DNA from five leaves from one transformed Talana plantlet produced the PCR-amplified 558 bp fragment (Fig. 4.9, lanes 5, 7-10). The 558 bp fragment was also present in the pBI101/*pat* DNA (Fig. 4.9, lane 10) and 1.3 kb chimeric gene construct (Fig. 4.9, lane 2), as expected. No fragment was present in the negative controls (Fig. 4.9, lanes, 3-4), as expected.

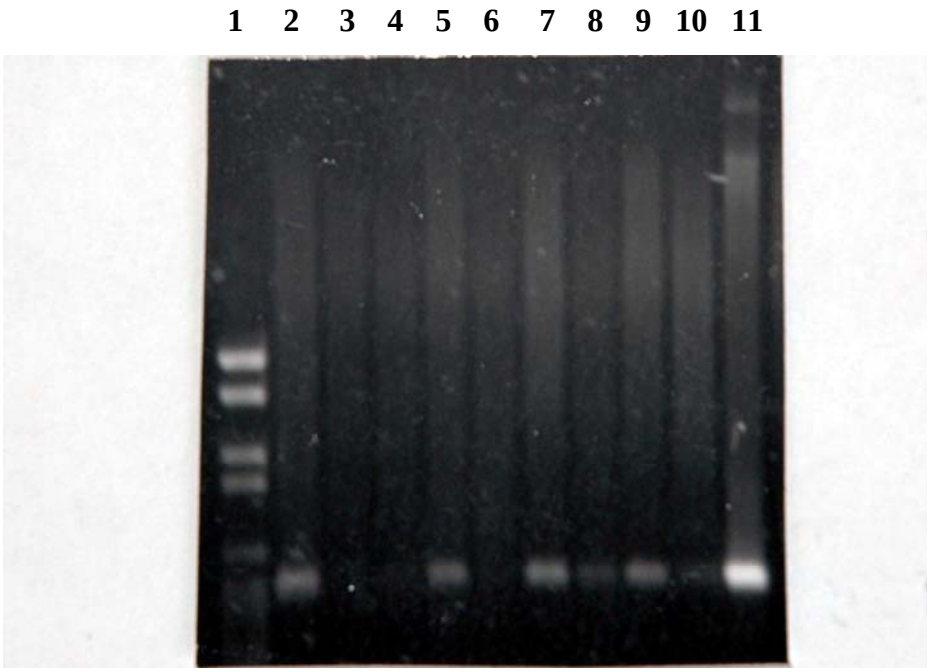

Figure 4.9: Amplification of the 558 bp *pat* coding region in transgenic Talana leaves from one transformed plant. Samples of 25 µl were subjected to 30 cycles of amplification. Two microlitres of each sample were resolved on a 0.8% agarose gel and visualised by ethidium bromide fluorescence.

Lanes: (1) molecular weight marker VI; (2) positive control - 1.3 kb chimeric gene construct negative control – non-transformed soybean DNA; (4) negative control - water sample; (5-10) transgenic Talana leaves from one transformed plant; (11) positive control - pBI101/*pat* DNA.

4.4 Discussion

4.4.1 Evaluation of South African soybean cultivars for susceptibility to *Agrobacterium*

Screening for *Agrobacterium* susceptibility at the whole plant level proved fruitful in identifying soybean cultivars, Talana and Ibis, capable of forming a compatible reaction with the pathogen. The data represented here confirms that Talana and Ibis are hosts for *A. tumefaciens* sis 43. The variation in percent visual tumour formation and tumour size among the two soybean cultivars observed in this study, in response to *Agrobacterium* challenge, has also been observed by others when screening different soybean cultivars for response to *Agrobacterium* (Pedersen *et al*., 1983; Owens and Cress, 1985; Byrne *et al*., 1987; Delzer *et al*., 1990; McKenzie and Cress, 1992; Bailey *et al*., 1994; Donaldson and Simmonds, 2000). The cultivar Peking was found to be highly responsive in previous studies (Owens and Cress, 1985; Kudirka *et al*., 1986; Byrne *et al*., 1987) but there has been no report on the response of the cultivar Talana, tested in this study. McKenzie and Cress (1992) reported on the tumourgenic response of Ibis and found that in greenhouse plants 1-13 tumours formed per 100 inoculation sites. In this study tumours on Ibis developed at 70 % of the inoculation sites and one to four small tumours were present at the inoculation sites.

In this study, Talana was shown to be more responsive/susceptible to *A. tumefaciens* sis 43 than Ibis, with 85 % of inoculations resulting in crown galls for Talana compared to 70 % for Ibis. Compared to crown galls on many other

dicotyledonous plants, soybean galls were small (< 3 mm) and slow growing. These results are in accordance with the results reported by Owen and Cress (1985). The fact that the wounded plant tissues inoculated with the virulent *Agrobacterium* formed neoplastic growths indicated that integration of T- DNA has occurred. Integration of T-DNA has two major effects: first, it leads to the development of a tumour through an increase in growth hormone biosynthesis by the transformed plant cells and secondly, it elicits the production by these transformed cells of specific secondary metabolites, called opines (Fortin *et al*., 1992). Various researchers (Pedersen *et al*., 1983; Owens and Cress, 1985; Byrne *et al*., 1987) have demonstrated T-DNA integration into plant DNA tumours on soybean (and the presence of several copies of integrated T-DNA in the plant chromosome) by opine tests and by molecular hybridization of restricted cell DNA with labelled T-DNA fragments.

In this study, tumours incited in the cultivars Talana and Ibis were 3 mm or less in diameter five weeks after inoculation. This compares favourably to the reported tumour size (3 mm) after inoculation of Ibis and other soybean cultivars (A5308, Duiker, Edgar, Highveld-Top, Forrest, Hutton, Impala, PNR577G, Ransom and Columbus) with a hypervirulent *A.tumefaciens* strain A281 (McKenzie and Cress, 1992.) In this study, the tumourgenic response of soybean was less than that of sunflower. The smaller tumour size of soybean may have resulted from fewer cells initially being transformed by *A. tumefaciens*. The low transformation efficiency in soybean, as indicated by the small number of tumours formed, supports this view. However, the genotype-dependent hormone metabolism of the

plants could have also played a role in the slow development of tumours in soybean. The tumours on sunflower plants developed more rapidly (within 10 days) than those on soybean (3 weeks) and were much larger in size. These findings are in keeping with the results reported by other researchers (Owens and Cress, 1985; McKenzie and Cress; 1992) who found that differential responses of soybean and sunflower genotypes to virulent *Agrobacterium* strains.

The expression of several traits that affect the interaction of the host plant with *Agrobacterium* isolates include traits that affect the initial survival of *Agrobacterium* in the wounds, the age of the explant and type of explant, the plant genotype and genetic composition of the infecting *Agrobacterium* strain, the subsequent binding of bacteria to the cell wall, cell wall fragment, the transfer of DNA into the host cell, the integration of DNA into the plant chromosome and the endogenous phytohormone levels in the stem that could enhance or inhibit tumour growth and possibly determine the age at which galls begin to senesce (Owens and Cress, 1985; Byrne *et al*., 1987; Rech *et al*., 1989). Successful infection of a host with *Agrobacterium* requires a compatible reaction between host plant components and bacterial components (Byrne *et al*., 1987). Anderson and Moore (1979) demonstrated differences in infectivity by *Agrobacterium* strains for a particular host species and the differences obtained may reflect differences in susceptibility of the host, the virulence of the pathogen, or the interaction of the two.

In this study all soybean plants grown in the growth chamber and grown *in vitro* developed tumours. The tumours on the growth chamber-grown plants tended to form masses of small galls as described by Owens and Cress (1985). The findings in this study are in contrast with the results of McKenzie and Cress (1992) who reported that the incidence of infection was low, especially in greenhouse grown seedlings. In their study, in many cases, tumours could only be induced under humid conditions. Cultivars that did not form tumours in the greenhouse were able to develop them in a more humid atmosphere inside flasks. Several researchers (Pedersen *et al*., 1983; Owens and Cress, 1985; McKenzie and Cress, 1992) suggested that a genotype x environment interaction affects the host's response (tumour formation) to the pathogen. Owens and Cress (1985) reported on wide genotypic variation in susceptibility to infection with Ti bearing *Agrobacterium*. The tumourous growths induced by *A. tumefaciens* strains have been shown to be primarily due to the activity of three T-DNA linked oncogenes, namely iaaM, iaaH and iptZ. The co-operative expression of these genes leads to a deregulated production of phytohormones that in turn induces neoplastic proliferation of the transformed cells as well as of their neighbouring cells (Zambryski *et al*., 1989).

The controlled growth chamber conditions in this study and the covering of the inoculation sites to prevent desiccation were found to promote the development of the tumours and allowed reproducible isolation of tumour tissue. These results are in accordance with the results reported by Pedersen *et al*. (1983) who found that the sensitivity of dehydration in the initial stage of tumour formation may be

a reason why soybean has not been reported to respond to infection with *A. tumefaciens*. Pedersen and his co-workers (1983) also confirmed that with no protection of the wound site, the soybean cultivar Mandrian responded to *A.tumefaciens* infection in a weakly susceptible fashion.

The tumours from Talana and Ibis and the sunflower were isolated and cultured *in vitro* as sterile callus on hormone-free medium. Growth of crown galls cells in culture is independent of phytohormones (Pedersen *et al*., 1983) and hence the T-DNA seems to be stably integrated into the soybean DNA and expressing the genes for maintenance of phytohormone independent growth. These results confirm the reports by Pedersen *et al*. (1983) and Owens and Cress (1985) who reported proliferated unorganised growth of callus from soybean (cultivars Mandrian and Peking) tumours on hormone-free medium.

4.4.2 Determination of the concentrations of antibacterial and selection agents for use in transformation experiments

When attempting transformation with *Agrobacterium tumefaciens* one of the most important steps is the selection of transformed cells from an abundance of non-transformed cells. Antibiotics are widely used in genetic transformation technology to select transgenic tissues and/or to eliminate *Agrobacterium* from the cultures when its presence is no longer required (Estopa *et al*., 2001). Eliminating *A. tumefaciens* from cultures is important, because microbial contaminants in cultured plants can reduce multiplication and rooting rates or induce plant death

(Cassells, 1991). Moreover, the elimination of *Agrobacterium* in transgenic plants is a pre-requisite in preventing the possibility of gene release when these plants are transferred to the soil (Barrett *et al*., 1997).

Carbenicillin, cefotaxime and ticarcillin, belonging to the β-lactam group, are the antibiotics most commonly employed to eliminate *Agrobacterium* from cultures (Estopa *et al*., 2001). These antibiotics inhibit bacterial cell wall synthesis (Holford and Newbury, 1992). The effect of these antibiotics have been studied on different plant species (Estopa *et al*., 2001). The levels of antibacterial agents included in the regeneration medium for soybean had no detrimental effect on the regeneration response. Similarly, Draper *et al*. (1988) reported that cefotaxime and carbenicillin had no effect on normal development of callus from various plant species. Chevreau *et al*. (1997) reported that cefotaxime did not have negative effects on bud regeneration when pear leaf explants were cultured.

In order to evaluate the potential use of kanamycin and PPT as selection agents (double selection) for use in the selection of transformed soybean explants, a determination of the minimal concentrations of selection agents that inhibited shoot and root regeneration was determined. All concentrations of PPT tested produced visual injury symptoms of chlorosis for Talana and Ibis shoot apical meristems and they became necrotic. Talana and Ibis shoots became necrotic and did not produce roots when 1 mg/l PPT was included in the B5 rooting medium. From this result, 1 mg/l PPT was chosen for the selection of *Agrobacterium*-transformed herbicide resistant soybean shoot apical meristems. Similarly, Zhang

et al. (1999a) and Olhoft and Somers (2001) used 1.33-5 mg/ PPT for selection of transformed soybean (cultivars A3237 and Bert, respectively) cotyledonary node cells. Zeng *et al*. (2004) reported using 8 mg/l glufosinate ammonium (PPT) for the first and second shoot initiation stages from transformed cotyledonary node cells of the soybean cultivar Williams 82 and 3-4 mg/l during shoot elongation. Li *et al*. (2000) used 20 mg/l glufosinate ammonium when selecting for transformed lupin shoots from shoot meristems which had been transformed with *Agrobacterium*. Shu *et al*. (2005) used 1 mg/l PPT when selecting for transformed perennial grass plantlets. Other researchers have reported that transformed shoot apices of rice cultured on medium containing 0.5 or 1.0 mg/l PPT survived (Park *et al*., 1996).

In this study, the level of sensitivity to kanamycin was 100 mg/l for soybean shoot apical meristems. Similarly McKenzie and Cress (1992) used 100 mg/l kanamycin for selection of transformed hypocotyls and 200 mg/l kanamycin for transformed cotyledons of the soybean cultivar Forrest. Kanamycin is one of the most widely used selection agents for plant transformation. The corresponding resistance gene (*npt* II) encodes an aminoglycoside 3'-phosphotransferase, which inactivates kanamycin by phosphorylation (Bowen, 1993).

To be of use, the selective agent must exert stringent pressure on the plant tissue concerned. The effectiveness of a particular compound as a selective agent can also vary with the type of target explant or tissue to be selected, genotype and species (D'Halluin *et al*., 1992; Rathore *et al*., 1993; Estopa *et al*., 2001), with

escapes (false-positive shoots) being one of the major problems. The level of difference, type and size of explant stressed on selection medium all affect the usefulness of any selectable marker gene (Draper *et al*., 1988). Double selection used in transformation protocols is more efficient in eliminating potential escapes (Chen and Punja, 2002).

4.4.3 Transformation of shoot apical meristems by *Agrobacterium*-mediated transfer

In recent years, procedures for the transformation of soybean cultivars by *Agrobacterium* have been reported (Clemente *et al*., 2000; Donaldson and Simmonds, 2000; Yan *et al*., 2000; Ke *et al*., 2001; Olhoft and Somers, 2001; Zeng *et al*., 2004; Shan *et al*., 2005), but the transformation frequencies were low (10% and less) and the plants recovered were often chimeric (Parrot *et al*., 1989; Meurer *et al*., 1998). Transformation of soybean shoot apical meristems by particle bombardment has been reported by McCabe *et al*. (1988) and Sato *et al*. (1993), but a reproducible method for the transformation of soybean (cultivar Talana) shoot apical meristems using *Agrobacterium* has not previously been described. In this study, a simple and rapid method for transforming soybean shoot apical meristems is described. This work presents the first reported method for the transformation of shoot apical meristems of the South African grown soybean cultivar Talana.

The choice of explant for *Agrobacterium*-mediated transformation is of extreme importance for successful transformation (D'Halluin *et al*., 1992). Shoot apical meristems were used as the starting material in this study because whole plants could be regenerated from shoot apical meristems of Talana and Ibis (Chapter 3). The shoot apical meristem of plants generates whole plant shoots *in situ* in even very recalcitrant plant species and thus possess considerable potential for genotype independent gene transfer to recalcitrant species (Potrykus, 1990). Using shoot apical meristems for transformation has several advantages over using immature embryos. First is the ability to obtain target tissues for transformation from dry seeds; the necessity to grow donor plants under controlled conditions is eliminated (Zhong *et al*., 1998). The second advantage is the direct vigorous regeneration of shoots from shoot apical meristems. Shoot regeneration is direct, relatively simple and needs less time to regenerate large numbers of plants (Saeed *et al*., 1997). Thirdly, plants regenerated from shoot apices are true to phenotype with low incidence of somaclonal variation and chromosomal abnormalities (Bajaj, 1998). The fourth advantage is that the shoot apex explant has few genotype limitations and is considered as more appropriate because meristematic cells are programmed for direct shoot organogenesis without an intervening callus or de-differentiation stage (Zhong *et al*., 1998; Zapata *et al*., 1999b). One of the disadvantages of using shoot apical meristems for transformation is that the genotype of a seedling is unknown and commercially the application of transformation is to further explore superior (known) genotypes. Another disadvantage is that the primary transformants will always be chimeric (Lowe *et al*., 1995; Zhong *et al*., 1996). The relatively stability of chimeric shoots

depends on the original position of the transformed cell(s) and the competition between transformed and non-transformed cells (Marcotrigiano, 1990).

Meristem methods have been used successfully in *Agrobacterium*-mediated transformation of many plants like corn (Gould *et al*., 1981b), lupin (Li *et al*., 2000), pear (Matsuda *et al*., 2005), rice (Park *et al*., 1996) and cotton (Zapata *et al*., 1999a; Satyavathi *et al*., 2002).

In order to enhance transformation rates by *Agrobacterium*-mediated DNA transfer, improvements have been made in the delivery of the bacterium (Bidney *et al*., 1992), and vectors have been modified to provide constitutive expression of *vir* genes (Ishida *et al*., 1996). Although transformation rates have been significantly improved using these modifications, increases in efficiency are still needed. The response of plants to wounding is an important factor in the interaction between *Agrobacterium* and plants (Potrykus, 1991). There have been reports were frequency of transformation mediated by *A. tumefaciens* can be greatly enhanced by first generating microwounds in the target tissue through high-velocity microprojectile bombardment with uncoated particles (Bidney *et al*., 1992; Knittel *et al*., 1994; Brasileiro *et al*., 1996) or particles coated with *Agrobacterium* (Cordero de Mesa *et al*., 2000), shaking with glass beads (Grayburn and Vick, 1995; Alibert et al., 1999) or with carborundum (Cheng *et al*., 1996), sonication (Santarem *et al*., 1998) and scratching with sandpaper (Hoshi *et al*., 2004). Future studies to improve the transformation frequency of commercially grown South African soybean cultivars could include some of the

above-mentioned protocols and the addition of cysteine into the media during co-cultivation with *Agrobacterium* to increase T-DNA delivery (Olhoft and Somers, 2001; Zeng *et al*., 2004).

Park *et al*. (1996) and Li *et al*. (2000) wounded the shoot apical meristems of lupin and rice, respectively, with a fine needle, to place bacteria in the shoot meristematic region. Their results indicated that extra wounding treatment with the hypodermic needle through the surface of the shoot apex may enhance stable transfer of T-DNA into the cells T-DNA into the cells because access for *Agrobacterium* to a cell surface recognition site is provided and the T-DNA can be integrated into the plant genome and transcribed into messenger RNA (Draper *et al*., 1988). Based on their results, the shoot apical meristems of Talana and Ibis, in this study, were wounded with a needle and the *Agrobacterium* placed into the shoot apical meristems prior to incubation with *Agrobacterium*. This is the first report on inoculating of shoot apical meristems of soybean. Two transformed Talana shoot apical meristems produced shoots for more than two months on medium containing 1 mg/l PPT, indicating that they were undamaged and transformed and that the *pat* gene was producing sufficient amounts of PAT to confer PPT-resistance under the selection conditions.

In this study, the overall transformation efficiency from the initial 100 Talana explants to transformed plants in tissue culture was low (1.06%). When the transformed plants were transferred to potting mixture and acclimatized, they died and so no established plants were obtained and so the frequency of regenerated

transgenic plants established in soil could not be calculated. Other researchers have also reported low soybean transformation efficiencies from *Agrobacterium*-mediated transformation of soybean cotyledons and cotyledonary nodes. Hinchee *et al*. (1988) reported 0.7-2% glyphosate resistant soybean (cultivar Peking) shoots produced from the transformation of cotyledon explants by *Agrobacterium*. Zhang *et al*. (1999a) reported glufosinate resistant soybean (cultivar A3237) plants recovered at frequencies up to 3% following transformation of cotyledonary nodes with the *bar* gene. Donaldson and Simmonds (2000) reported 0.4% transformants following transformation of 6.8% cotyledonary nodes of the soybean genotype AC Colibri. Di *et al*. (1996) obtained a transformation frequency of 1.25% for cultivar Fayette however several of the plants recovered did not transmit the transgenes stably to the progeny. Zeng *et al*. (2004) transformed 1,318 cotyledonary nodes (of which 578 regenerated) and reported a final recovery frequency of transgenic soybean plants (cultivar Williams 82), defined as greenhouse established fully-grown plants, to be 5.4%. An increase in soybean cotyledonary transformation has been reported by Olhoft *et al*. (2003). These researchers obtained a transformation frequency of 16.4% following transformation of soybean (cultivar Bert) cotyledonary nodes. The results represented in our study demonstrate that shoot apical meristem tissues from Talana seedlings are competent for *Agrobacterium*-mediated transfer. Future transformation studies need to include protocols for improving acclimatization of transgenic soybean plants.

Shoot meristems of soybean have been transformed by particle bombardment (McCabe *et al*., 1988; Sato *et al*., 1993). Sato *et al*. (1993) reported that chimeric, transgenic shoot primordia and plantlets of the cultivar Williams 82 were produced. No non-chimeric, transgenic plantlets were produced. Only 0.4% of nearly 3000 regenerated plantlets contained *GUS* positive sectors in their stems at the time of rooting and none of the rooted chimeric plants tested at the five-trifoliate leaf stage produced *GUS* positive leaves. The *GUS* positive sectors that were detected in regenerated shoot primordia and plantlets were primarily superficial, indicating that both non-transformed and transformed cells are involved in shoot initiation (Sato *et al*., 1993). It is possible that only the epidermal and subepidermal cells were transformed in bombarded shoot tips since the majority of particles were found in the outermost two cell layers of the apex (Sato *et al*., 1993). McCabe *et al*. (1988) reported on the transformation of soybean cultivars Williams 82 and Mandarin Ottawa. Their experiments yielded approximately 2% transformed chimeric plantlets.

Other researchers have obtained transgenic plants from *Agrobacterium*-mediated transformation of the shoot apical meristem. Reported transformation efficiencies have also been low, as obtained in the present study. Gould *et al*. (1981b) reported a transformation frequency of 0.075% from shoot meristems of corn. Li *et al*. (2000) reported an overall transformation frequency of lupin, as determined at the T1 generation, of 0.05%-0.75%. Matsuda *et al*. (2005) reported a transformation frequency of 4.8% for pear shoot meristem-explants. Park *et al*. (1996) reported a transformation frequency of 2.8% when rice shoot apical

meristems were transformed using *Agrobacterium tumefaciens*. Satyavathi *et al*. (2002) and Zapata *et al*. (1999a) reported 1.47% and 0.8% regenerated cotton plants, respectively, which were obtained from transformed shoot apical meristems of cotton. Transformation frequencies obtained by *Agrobacterium*-mediated transfer differ between plant species and factors such as wound induction and selection protocols all affect transformation efficiencies.

May *et al*. (1995) reported that when shoot apical meristems of banana were transformed with *Agrobacterium*, 40% of the tissues which formed shoots on kanamycin demonstrated vigorous root growth when transferred to rooting medium containing kanamycin. They reported that the remaining 60% of the shoots were chimeric. Gould *et al*. (1981b) also reported on possible chimeras produced when shoot apical meristems of corn were injected with *Agrobacterium*. In this study, only eight transformed shoots out of fifteen obtained from the shoot apical meristems of Talana rooted in the presence of kanamycin and PPT and formed plantlets. This data possibly indicates that the remaining shoots were chimeric, and did not contain meristematic tissues that would give rise to PPT-resistant roots.

PCR confirmed the presence of the *pat* gene in the eight transformed Talana plants. DNA from all leaves tested contained the *pat* gene indicating that there were no chimeric leaves on the transformed plantlets. Rooting in the presence of selection agents therefore eliminated the possibility of chimeric shoots that may have been formed when the shoot apical meristems were transformed. Due to the

fact that the transformed Talana plants died and were not acclimatized in the growth chamber, no seed was produced and no more leaves were available for extraction of plant DNA. As a result, no Southern blotting or herbicide application experiments could be performed. Further molecular analysis is needed to determine integration, expression and stability of the *pat* gene in transformed shoot apical meristems and in the progeny. Herbicide application studies are needed to establish the percentage of herbicide tolerant plants.

The 0% transformation efficiency for Ibis shoot apical meristems obtained in this study was probably attributed to inefficient transformation of regenerable cells, and/or poor selection or survival of such cells, and not poor susceptibility to *Agrobacterium*, since Ibis was found to be susceptible to *Agrobacterium*. Similarly, Donaldson and Simmonds (2000) reported that the significant obstacle for recovery of transgenic soybean plants using the cotyledonary node method is the inefficient targeting of transformation in the cotyledonary node and not poor susceptibility to *Agrobacterium*. Regeneration-competent cells of the cotyledonary node are probably few, and they may have low transformation competency at the time of co-culture. The recovery of transgenic plants may also involve cultivar regeneration potential on selective media (Meurer *et al*., 1998; Murray *et al*., 2004).

It has been reported that during selection of transformants on medium containing selection agents, the majority of the cells in the explant die. Such dying cells may release toxic substances (such as phenolics) that in turn may impair regeneration

of transformed cells. In addition, dying cells may form a barrier between the medium and the transgenic cells preventing or slowing uptake of essential nutrients (Joersbo and Okkels, 1996). The overall effect of necrotic tissue during selection is presumably reduced mitotic activity of the transformed cells resulting in less transgenic shoots emerging from the explants (Joersbo and Okkels, 1996).

The production of transgenic soybean plants using *Agrobacterium tumefaciens* was recently improved by increasing the transformation of cotyledonary node cells through the addition of L-cysteine to medium used for the co-cultivation of *Agrobacterium* with the cotyledonary node explant (Olhoft and Somers, 2001; Zeng *et al*., 2004). In the study conducted by Olhoft and Somers (2001), a transformation frequency of 0.9% was obtained in the non-cysteine-treated explants. By comparison, the cysteine-treated explants produced transgenic plants at a frequency of 2.1%. There was also a notable decrease in enzymatic browning on wounded and infected soybean explants co-cultivated with cysteine (Olhoft and Somers, 2001). Since both *Agrobacterium* and infection and tissue browning were affected, it is possible that cysteine interacts with the plant's response to wound and pathogen infection during co-cultivation, resulting in an increase in T-DNA delivery (Olhoft and Somers, 2001).

It has been reported that in the presence of a low concentration of acetosyringone, sugars such as glucose and galactose act synergistically as virulence-inducing agents during infection by *Agrobacterium* (Cangelosi *et al*., 1990; Fortin *et al*., 1992). Khan *et al*. (1994) also reported on the glucose and acetosyringone

requirement in the co-cultivation medium for efficient gene transfer in clover. Similarly, Santarem *et al*. (1998) reported that the addition of 100 µm acetosyringone to the co-culture medium enhanced transient expression in transformed immature soybean cotyledons. Trick and Finer (1998) added 100 µM acetosyringone to the maintenance medium when transforming embryogenic suspension cultures with *Agrobacterium*. Olhoft and Somers (2001) added 200 µM acetosyringone to the co-culture medium when transforming soybean cotyledonary node cells. Based on these findings, the soybean explants in this study were co-cultivated in the presence of glucose and acetosyringone. The presence of acetosyringone and glucose in the co-cultivation medium possibly had a positive effect on the transfer of the *pat* gene into the Talana shoot apical meristems, hence the regeneration of the Talana shoot apical meristem explants on medium containing kanamycin and PPT. Future transformation studies without acetosyringone and glucose will need to be done to determine transformation efficiency with and without these agents.

In this study, shoot apical meristems were inoculated with the *Agrobacterium* culture immediately after excision to allow efficient transformation of the plant cells. Kudirka *et al*. (1986) reported that Peking explants inoculated 4 h after excision showed reduced frequency of transformation. Excision stimulates wound-associated cell divisions that make the plant cells competent for transformation as well as providing access for the bacterium to a cell surface recognition site. Plant cells must be undergoing genome replication and can be easily accessible to *Agrobacterium* to achieve transformation (Draper *et al*.,

1988). The response of plants to wounding is an important factor in the interaction between *Agrobacterium* and plants (Potrykus 1991).

In this study, transgenic herbicide resistant Talana plantlets were obtained within five months. Once the transformed plantlets were transferred to sterile potting mixture in tissue culture flasks, the plantlets lost their leaves and died. When non-transformed plants were subjected to exactly the same method, they continued to grow into mature plants. The reason that mature transgenic soybean plants were not produced was not due to the tissue culture protocol, but possibly the result of transformation. Similarly, McKenzie and Cress (1992) reported that no mature transgenic Forrest soybean plants were obtained when cotyledons were transformed with *Agrobacterium* because of problems acclimatizing the transgenic shoots. The plantlets failed to grow unless their stems had elongated sufficiently before rooting. Future work needs to be done to increase recovery of mature transgenic plants in the soil.

The results represented here demonstrate that shoot apical meristems of soybean can be used for *Agrobacterium*-mediated transfer. However, it should be noted that no transformation system is applicable to all genotypes, as demonstrated in this study. Transformed Talana shoot apical meristems were obtained but no transformed Ibis shoot apical meristems. In future studies more commmerically grown South African soybean cultivars need to be evaluated to determine whether this transformation protocol is genotype-dependent.

4.5 Conclusions

In conclusion, the commercially grown South African soybean cultivar Talana has been genetically transformed with the *pat* gene by a rapid and simple *Agrobacterium*-mediated transformation protocol. Eight transgenic Talana plants were produced from 100 explants, giving an overall transformation efficiency of 1.06%. The results obtained in the present study are promising, since data on transformation of shoot apical meristems of South African cultivars of soybean are not available. The presence of weeds in soybean fields is one of the main causes of productivity loss and the generation of a herbicide-resistant soybean variety is a significant step towards the genetic improvement of soybean. To the best of our knowledge, this is the first report of *Agrobacterium*-mediated soybean transformation using shoot apical meristems as a target explant. The present study highlights the feasibility of obtaining transgenic soybean using the shoot apical meristem and *Agrobacterium*-mediated DNA transfer. This method may possibly be extended to other soybean cultivars and can be applied to transform soybean with genes that have economical importance. The transformation methodology developed in this study will thus be useful for future soybean crop improvement programmes in South Africa.

CHAPTER 5

The effect of various cytokinins on the induction of multiple shoots and plant regeneration in commercially grown South African cotton cultivars.

Sections of this chapter have been presented as a paper at the World Cotton Research Conference-2, Divani-Caravel Hotel, Athens, Greece, 6-12 September 1998.

C. McNaughton and M.E.C. Rey. Development of an efficient regeneration and transformation system for commercially grown South African cotton cultivars.

This chapter will be submitted for publication in Plant Cell, Tissue and Organ Culture.

C. McNaughton-Pascoe and M.E.C. Rey. The effect of various cytokinins on the induction of multiple shoots and plant regeneration in commercially grown South African cotton cultivars.

Abstract

Efficient and reproducible plant regeneration protocols are essential for successful transformation of cotton (*Gossypium hirsutum*). In this study, a rapid, efficient, simple, reproducible, and possibly genotype dependent method of *in vitro* propagation using shoot apical meristem explants has been developed for commercially grown South African cotton cultivars Sabie, Palala, LRCC 101 and 107/1. Shoot apical meristems (0.5-1 mm in length) from 5-7 day old seedlings were cultured on MS medium supplemented with 0.0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.5 and 5.0 mg/l kinetin, zeatin, BA and TDZ, individually, and the percentage of shoot apical meristems producing multiple shoots recorded after four weeks of culture. The highest percentage (90-100%) of shoot apical meristems of all four cultivars producing multiple shoots was on medium containing either kinetin or zeatin. The concentrations of kinetin which produced the highest percentage (100%, 93.3%, 99.3% and 100% respectively) of shoot apical meristems of Sabie, LRCC 101, 107/1 and Palala producing multiple shoots, were 0.1 mg/l, 0.05 and 1.0 mg/l, 1 mg/l and 0.5 mg/l, respectively. The number of shoots produced per meristem at these concentrations was 5-6 for Sabie and Palala, 3-4 for LRCC 101, 4-5 for 107/1. The concentrations of zeatin which produced the highest percentage (95.3%, 99.3%, 99.3% and 94% respectively) of shoot apical meristems of Sabie, LRCC 101, 107/1 and Palala producing multiple shoots, were 0.01 mg/l, 0.05 mg/l, 0.01 mg/l and 0.05 mg/l, respectively. The number of shoots produced per meristem at these concentrations was 3-5 for Sabie, LRCC 101 and 107/1 and 3-4 for Palala. The highest percentage (85.3%, 82%, 66% and

80.6%, respectively) of shoot apical meristems of Sabie, LRCC 101, 107/1 and Palala producing multiple shoots was on medium containing BA at concentrations of 0.05 mg/l for Sabie and LRCC 101, 0.10 mg/l for 107/1 and 0.01 mg/l for Palala. The number of shoots produced per meristem at these concentrations was 3-4 for Palala and LRCC 101 and 3-5 for Sabie and 107/1. The highest percentage of shoot apical meristems producing multiple shoots was at different concentrations of TDZ for the different cultivars: 0.01 mg/l for Sabie (80.6%), 0.01 mg/l for LRCC 101 (46%), 0.05 mg/l for 107/1 (55.3%) and 0.05 mg/l for Palala (66%). Media containing 0.5 mg/l or more TDZ caused loss of apex organization and promoted callusing of the shoot meristem for cultivars Sabie, 107/l and Palala. The callus production was, in general, accompanied by the production of phenolic compounds. The shoots that were produced from the explants (2-3 per meristem for Palala and 107/1 and 2 per meristem for Sabie and LRCC 101) after four weeks at the lower concentrations of TDZ were small (>0.5 cm), did not elongate and eventually showed signs of chlorosis and became necrotic. TDZ was therefore concluded to be an ineffective cytokinin for shoot production from shoot apical meristems of the four cotton cultivars. Shoot regeneration from shoot apical meristems appeared to be genotype dependent at the different concentrations of cytokinins. One hundred healthy green shoots (2-3 cm) for each cultivar were excised and transferred to MS medium containing 0.1 mg/l kinetin which allowed for elongation (to 3-5cm) and multiplication of shoots before transfer to rooting medium. Root initiation from 16 shoots (for each rooting media tested) of all four cotton cultivars did not occur on half-strength MS medium with or without 0.05 mg/l and 0.1 mg/l NAA or IBA. These hormone

treatments resulted in tissue mortality. Rooting of elongated Sabie, Palala, LRCC 101 and 107/1 shoots (3-5 cm) occurred on full strength MS medium containing 0.1 mg/l kinetin. Rooted plants were maintained on this medium for four weeks. The shoots elongated and a well developed rooting system developed. The overall percentage of rooted shoots from the isolated shoots was 90% (18/20) for Sabie, 80% (16/20) for LRCC 101 and 70% (14/20) for Palala and 107/1. These plantlets (5-8 cm) were transferred to sterile potting mixture and placed in the growth chamber for further growth. These plantlets grew and after four months, they bore flowers and bolls and produced viable seed under growth chamber conditions. All regenerated plants that were advanced to soil were phenotypically normal and appeared morphologically similar to seed-derived cotton plants. The overall frequency of regenerated cotton plants per meristem was 90% for Sabie, 74.6% for LRCC 101, 69.5% for 107/1 and 70% for Palala. This shoot apical meristem-based regeneration method can make a wider range of cotton germplasm accessible to improvement either by particle bombardment or *Agrobacterium*-mediated gene transfer.

5.1 Introduction

Cotton [*Gossypium* (*G.*) *hirsutum*] is a major fibre crop worldwide and its economic importance is increasing annually (Reiger *et al*., 1986; John, 1997; Ganesan and Jayabalan, 2004; Li *et al*, 2004). Cotton is a multi-billion dollar industry, and therefore a vital agricultural commodity to both U.S. and global economies (Mishra *et al*., 1993). Cotton is an excellent natural source of textile

fibre with a potential multiproduct base such as hulls, oils, paper, fertilizers, linters and meal (Hemphill *et al*., 1998; Zhang *et al*., 2000b; Mishra *et al*., 2003; Ganesan and Jayabalan, 2004). The production, marketing, consumption and trade of cotton-based products further stimulates the economy, and based on revenues in excess of $100 billion generated annually in the U.S. alone, cotton is the number one value-added crop (Mishra *et al*., 1993).

Cotton is grown on 2.5 % of the arable land in five continents. It is cultivated and grown mainly under subtropical and tropical environmental conditions (Nobre *et al*., 2001). It is grown in more than 80 countries and has an annual production of 20 million tons (Li *et al*., 2004). Among the cotton producing countries, India ranks first in production and area in cultivation, providing 32% of the world's total area of cotton cultivation, followed by the U.S.A. (23%) and China (20%), and it has been estimated that 180 million people depend, either directly or indirectly, on the production of cotton for their livelihood (Benedict and Altman, 2001). Cotton production is of high economic importance in Africa. Cotton is grown on 2.5 million ha of the continent, most of it on small plots of less than 10 ha (Scott, 2004). In 2004-2005 South Africa cotton was grown on 36 303 ha of land in various regions by both commercial and small-scale farmers (http://www.cottonsa.org.za). Cotton production areas in South Africa are the Northern Cape-Lower Orange River and Griqualand West, North West-Rustenberg, Northern Province- Limpopo Valley, Loskop and Springbok Flats, Mpumalanga and KwaZulu-Natal (http://www.cottonsa.org.za).

Considerable attention has been paid to improving cotton plants through genetic engineering (Llewellyn *et al*., 1990; Nobre *et al*., 2001; Mishra *et al*., 2003). Cotton biotechnology hinges on two tightly interlaced processes-transformation and regeneration. Regeneration protocols are a major prerequisite for the production of transgenic plants, however, *in vitro* regeneration of cotton plants has been notoriously difficult and genotype has been shown to play an important role in successful plant regeneration (Trolinder and Xhixian, 1989; Rauf and Rahman, 2005). Efficient *in vitro* techniques for the regeneration of large numbers of plantlets from cotton are limited when compared to other major commercial crops (Kumria *et al*., 2003).

Embryogenesis and subsequent plant regeneration has been obtained in *G. hirsutum* from callus produced from immature tissues, namely cotyledons (Davidonis and Hamilton, 1983; Firoozabady *et al*., 1987; Ganesan and Jayabalan, 2004), immature embryos (Rangan *et al*., 1984), immature embryos and hypocotyls (Mitten, 1985; Shoemaker *et al*., 1986), seedling hypocotyls (Pannetier *et al*., 1997; Kumria *et al*., 2003; Ganesan and Jayabalan, 2004; Tohidfar *et al*., 2005) and cotyledonary leaf sections (Kumria *et al*., 2003). Somatic embryogenesis from mature tissues (leaf, petiole and stem) has also been reported (Gawel *et al*., 1986; Trolinder and Goodin, 1988a and b; Zhang *et al*., 2000b). Nobre *et al*. (2001) reported on the regeneration of embryogenic callus and plantlets from stomatal guard cells. More recently, Rauf and Rahman (2005) have reported on the induction of callus, embryo induction and germination in hybrids of upland cotton in relation to doses of growth regulators. They reported

that genotype x growth regulator level interaction caused considerable variation in the expression of regeneration responses, suggesting that determination of specific level of growth regulator concentration in the medium was necessary for a particular genotype to obtain optimum response. Genotype x explant source interaction was, however, relatively less important. Differences among genotypes for percent embryo induction was clearly evident. Other researchers have also reported somatic embryogenesis and plant regeneration in several cotton lines (Sakhanokho *et al*., 2005).

Although plant regeneration efficiency via somatic embryogenesis has recently been improved to 75.8% (Ganesan and Jayabalan, 2004), genotype-dependent response, a prolonged culture period, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, and lack of shoot elongation are the problems associated with cotton regeneration (Kumria *et al*., 2003). Other several problems have been highlighted during the regeneration of cotton from somatic embryos, including the secretion of secondary metabolites from the explants in the medium, browning of callus after a short period of culture, a low frequency of embryo maturation and abnormal somatic embryo germination (Ganesan and Jayabalan, 2004). The majority of the reports on *in vitro* regeneration of cotton via somatic embryogenesis pertain to either wild or Coker varieties of *G. hirsutum* that are not highly cultivated (Agrawal *et al*., 1997). Improvement of tissue culture methods to induce efficient regeneration in a genotype independent manner is desirable.

Earlier attempts to culture cotton protoplasts isolated from cotyledons or hypocotyl callus resulted in the formation of microcolonies or callus, but no plants were regenerated (Firoozabady and DeBoer, 1986; Saka *et al*., 1987). However, Peeters *et al*. (1994) has reported plant regeneration from cell suspension-derived protoplasts of *G. hirsutum* using feeder cells. The protoplast-to-plant development system reported (Peeters *et al*., 1994) might become an important component of cotton improvement through techniques such organ transfer and somatic hybridization. More recently, Sun *et al*. (2005) reported that fertile regenerated plants were obtained from protoplasts via somatic embryogenesis. A reproducible procedure for the isolation of epidermal strips and plant regeneration from stomatal guard cells of cotton has been reported by Nobre *et al*. (2001).

To circumvent the problems of somatic embryogenesis, organogenesis from various explants has been investigated. Cotton plants have been regenerated from cotyledonary nodes (Agrawal *et al*., 1997; Gupta *et al*., 1997), hypocotyl explants (Mishra *et al*., 2003), embryonic axes (Morre *et al*., 1998) and shoot meristems (Gould *et al*., 1991a; McCabe and Martinell, 1993; Saeed *et al*., 1997; Hemphill *et al*., 1998; Zapata *et al*., 1999a and b; Satyavathi *et al*., 2002).

Shoot regeneration from the shoot apical meristem is direct, relatively simple and is not prone to somaclonal variation and chromosomal abnormalities (Gould *et al*., 1991a). Efficient and reproducible plant regeneration systems producing fertile plants have been developed from shoot apices of aseptically germinated seedlings of sorghum (Zhong *et al*., 1998), castor (Sujatha and Reddy, 1998), pearl millet

(Devi *et al*., 2000), soybean (Kartha *et al*., 1981; Barwale *et al*., 1986; Sato *et al*., 1993) and pear (Matsuda *et al*. 2005).

Cytokinins in general favour shoot organogenesis in cultured tissues (Thorpe, 1993). Shoot organogenesis from the shoot meristem of cotton has been achieved using kinetin (Gould *et al*., 1991a; Saeed *et al*., 1997), 6-benzyladenine (McCabe and Martinell, 1993; Hemphill *et al*., 1998; Zapata *et al*., 1999b; Satyavathi *et al*., 2002), 2,4-dichlorophenoxyacetic acid (Saeed *et al*., 1997) and TDZ (Satyavathi *et al*., 2002). In some cases NAA and IAA were also added to the regeneration medium (Gould *et al*., 1991a; Saeed *et al*., 1997; Zapata *et al*., 1999b; Satyavathi *et al*., 2002). To our knowledge, there have been no reports on the effects of the cytokinin zeatin on shoot organogenesis from the shoot apical meristem of cotton. There have been no reports on the regeneration of commercially grown South African cotton cultivars Sabie, 107/1, Palala and LRCC 101.

Recent advances in genetic engineering provide an opportunity to modify commercially grown South African cultivars by producing transgenic plants. To establish a working system for genetically engineering cotton cultivars, efficient regeneration protocols are necessary.

In view of the economic importance of cotton in South Africa and the potential to improve commercially grown cultivars by genetic transformation, an efficient regeneration protocol utilizing the shoot apical meristem of commercially grown South African cotton cultivars Sabie, Palala, 107/1 and LRCC 101, was

developed. The results of a comparative study of shoot regeneration efficiency from the shoot apical meristems of four cotton cultivars (Sabie, Palala, 107/1 and LRCC 101) to different cytokinins [kinetin, 6-benzyladenine (BA), zeatin and thidiazuron (TDZ)] in culture and plant regeneration are presented. To our knowledge, this is the first report on the regeneration of commercially grown South African cotton cultivars from the shoot apical meristem. The knowledge gained from the present study has strengthened our understanding of *in vitro* morphogenesis in South African cotton cultivars. Application of this knowledge base should facilitate the generation of transgenics and other biological objectives.

5.2 Materials and methods

5.2.1 Explant preparation

Seeds of commercially grown South African cotton cultivars, Sabie, Palala, LRCC 101 and 107/1, were obtained from the Tobacco and Cotton Research Institute, Rustenburg, South Africa. The seeds were washed briefly in soapy water and surface sterilized in 2.6% (v/v) sodium hypochlorite solution containing three drops of Triton X-100 per 200 ml for 20 min. Following this step and under sterile conditions, the seeds were placed in 70% (v/v) ethanol for 2 min. The seeds were then thoroughly rinsed four times in sterile distilled water. Following the rinsing steps, five seeds were sown in sterile petri dishes containing 20 ml of MS medium (Appendix A), pH 5.8. The seeds were germinated under growth chamber conditions (25±2°C; 16:8 h light:dark photoperiod with light supplied at

an intensity of 35-40 μE m^{-2} s^{-1}) for 5-7 days. After 5-7 days, shoot meristems measuring 0.5-1 mm in length were aseptically isolated from the *in vitro* grown cotton seedlings.

5.2.2 Induction of multiple shoots from shoot apical meristems

This experiment was designed to compare the cytokinins kinetin, zeatin, BA and TDZ with respect to their effects on the shoot formation from the shoot apical meristems of cotton cultivars Sabie, 107/1, Palala and LRCC 101. Shoot apical meristems isolated from 5-7 day old seedlings were placed onto 20 ml MS medium containing 1 ml/l of 1000X MS vitamins (Sigma), 2% (w/v) sucrose (BDH), 0.7% (w/v) purified agar (Oxoid), pH 5.8. The cytokinins at the following concentrations: 0.0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 mg/l were added individually to the MS medium after autoclaving and 20 ml of media dispensed into sterile petri dishes. The petri dishes were sealed with parafilm and the cultures were maintained at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}. Explants were subcultured onto the same fresh medium at bi-weekly intervals. After four weeks of culture, the number of shoot apical meristem explants forming multiple shoots were counted. Formation of more than two shoots by an explant was considered as induction of multiple shoots (Agrawal *et al*., 1997). For each experiment, 50 explants were used for each concentration of cytokinin tested. This experiment was replicated three times. The averages of meristems producing multiple shoots was calculated and expressed as a percentage.

5.2.3 Statistical analysis

Standard errors from the means were calculated (Clarke and Cooke, 1989). Due to the fact that the dependent variable (the presence or absence of multiple shoots) was dichotomous and explanatory variables were present, a logistic regression model was used. The logistic regression was performed (SAS Enterprise Guide 3.0) to determine whether the cultivar, cytokinins kinetin, BAP, zeatin and TDZ and varying concentrations of these cytokinins had a significant effect on the production of multiple shoots from Talana and Ibis shoot apical meristems. A logistic regression regresses the natural logarithm of the odds ratio on the explanatory variables. A logistic regression models the dependent variable, whether or not multiple shoots are present, on the explanatory variables, concentration, cytokinin and cultivar using the count data (frequency of shoot apical meristems producing shoots) corresponding to the different combinations of these variables. If Pr>ChiSq [probability associated with the test statistic (Wald Chi-Square) assuming no effect] <0.05, the effect is considered significant.

5.2.4 Elongation of shoots

One hundred healthy green shoots (2-3 cm) for each cultivar were excised from the shoot apical meristems cultured on kinetin at the concentrations which gave the highest percentage of meristems producing multiple shoots. These shoots were transferred to MS medium containing 1 ml/l of 1000X MS vitamins, 2 % (w/v) sucrose, 0.7 % (w/v) purified agar and 0.1 mg/l kinetin (Sigma), pH 5.8, for

elongation before transfer to rooting medium. The regenerated shoots in tissue culture flasks (10 cm x 5 cm diameter) containing 30 ml of the above medium were incubated for 14 days at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}.

5.2.5 Rooting of shoots

For rooting of elongated shoots, various auxins were added to media and the potential to induce rooting assessed. The following media were assessed: half-strength MS medium (Appendix A) without any hormones, half-strength MS medium to which α-napthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) at 0.05 mg/l and 0.1 mg/l were added. Full strength MS medium (Appendix A) to which 0.1 mg/l kinetin was added (for continued elongation of the shoots) was also assessed for root induction from the elongated shoots. Sixteen elongated Sabie, 107/1, LRCC 101 and Palala shoots (3-5 cm) were placed onto each media, except for MS medium with 0.1 mg/l kinetin, onto which 20 shoots for each cultivar were placed. Elongated shoots were placed in tissue culture flasks (10 x 5 cm diameter) containing 30 ml of the above rooting media and were incubated at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}. Plantlets (5-8 cm) with adequate roots were removed from the culture vessels and cleaned with running tap water. After washing, the plantlets were then transferred to sterile potting mixture (Appendix C) in pots (12 cm x 14.5 cm diameter) and acclimatized, by enclosure with glass jars, in the growth chamber, which was maintained at a temperature of 25±2°C with a 16:8 h

light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}. Regenerated plants were watered with 300 ml water containing Multifeed (Plaaskem) (Appendix C) almost once or twice a week depending on the dryness of the soil. Subsequently glass jars were removed and regenerated plants watered daily. Multifeed was applied once a week. Visual observations (height of plant; shape, width and length of leaves; size, shape and colour of flowers, size of bolls) of regenerates were compared to seed-initiated plants.

5.3 Results

5.3.1 Effect of cytokinins on shoot formation from the shoot apical meristem

The effects of BA, TDZ, kinetin and zeatin on shoot organogenesis from shoot apical meristems of Sabie, 107/1, LRCC 101 and Palala were studied. Multiple shoots were directly induced from the shoot meristems of all cotton cultivars by all four cytokinins tested within two weeks in culture. The earliest shoot development was noticed after nine days in culture medium containing 0.01-1.0 mg/l kinetin. Shoots were concentrated at the apex and subsequent growth varied from simple shoot enlargement to multiplication at the base to form new shoots within two months. Shoot proliferation was maintained by bi-weekly transfers to fresh medium. The percentage of shoot meristems of Sabie, LRCC 101, 107/1 and Palala which produced multiple shoots was influenced by the cytokinin type and concentration (Figs. 5.1, 5.2, 5.3 and 5.4).

MS medium induced shoots from the shoot apical meristems of all cultivars after seven days. Green callus was initiated at the base of the shoot apical meristem inserted into the agar, and shoot development occurred from the shoot meristem. Shoots appeared normal, green and healthy as shown in Fig. 5.5. The percentage of shoot apical meristems producing multiple shoots ranged from 84-95.3% for Sabie, 88.6-96% for LRCC 101, 80-95.3% for 107/1 and 88-94% for Palala (Figs. 5.1, 5.2, 5.3 and 5.4). The number of shoots produced per meristem explant was 3-4 for Palala and 3-5 for Sabie, LRCC 101 and 107/1.

Shoot meristems from Sabie, 107/1 and Palala differentiated into multiple green shoots when 0.01-0.1 mg/l TDZ was added to the medium. LRCC 101 shoot meristems differentiated into multiple green shoots when 0.01-0.5 mg/l TDZ was added to the medium. The highest percentage of shoot apical meristems producing multiple shoots was at different concentrations of TDZ for the different cultivars: 0.01 mg/l for Sabie (80.6%), 0.01 mg/l for LRCC 101 (46%), 0.05 mg/l for 107/1 (55.3%) and 0.05 mg/l for Palala (66%) (Figs. 5.1, 5.2, 5.3 and 5.4). For the four test cultivars, media containing TDZ was not adequate for normal shoot development. The shoots that were produced from the explants were small (>0.5 cm), did not elongate and eventually showed signs of chlorosis and became necrotic (Fig. 5.6). The number of shoots produced per meristem explant was 2-3 for Palala and 107/1 and 2 for Sabie and LRCC 101. Media containing 0.5 mg/l

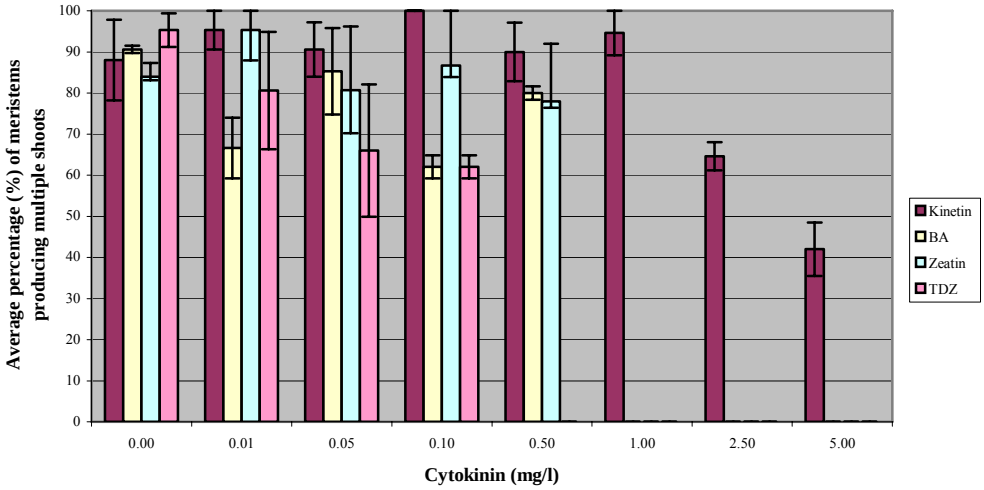

Figure 5.1: Average percentage of Sabie shoot apical meristems producing multiple shoots at different concentrations of cytokinins kinetin, BA, zeatin and TDZ. Values are the means of three replicates with 50 explants in each replication. Error bars represent the standard error from the mean.

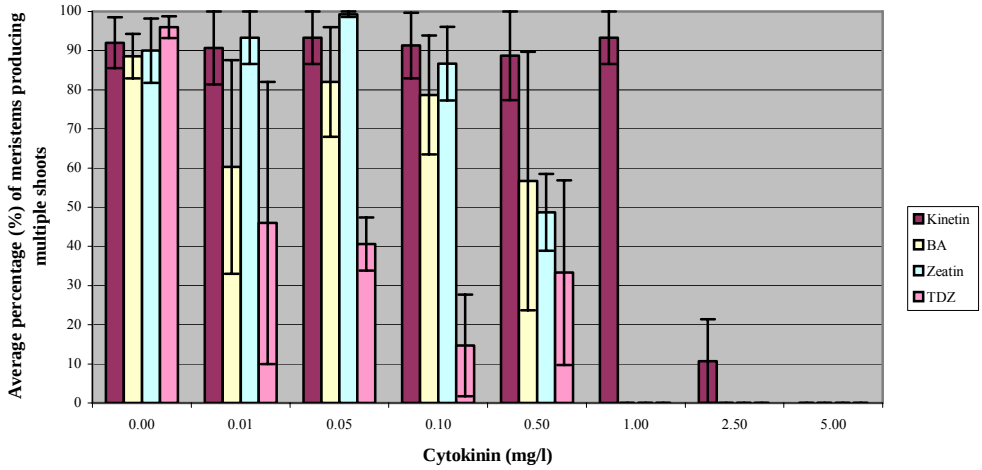

Figure 5.2: Average percentage of LRCC 101 shoot apical meristems producing multiple shoots at different concentrations of cytokinins kinetin, BA, zeatin and TDZ. Values are the means of three replicates with 50 explants in each replication. Error bars represent the standard error from the mean.

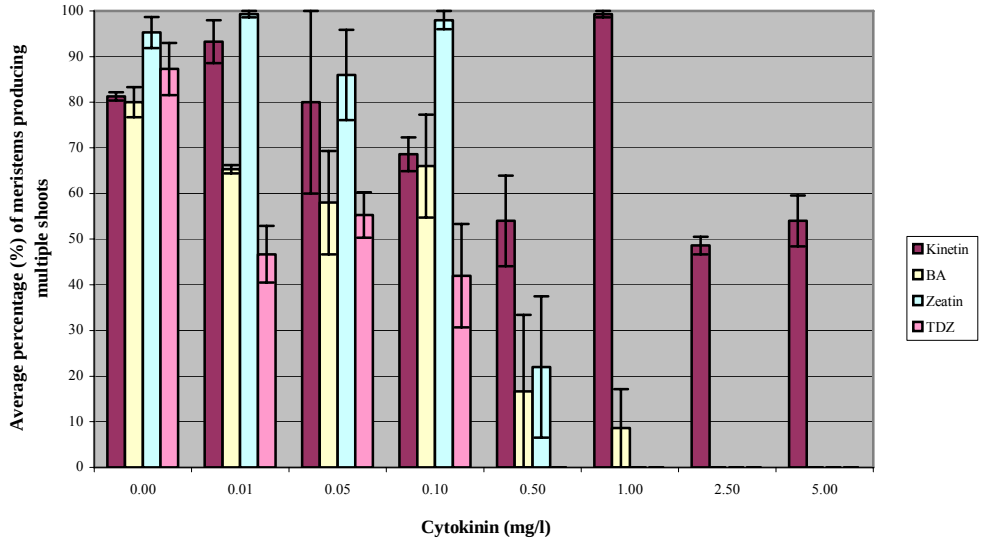

Figure 5.3: Average percentage of 107/1 shoot apical meristems producing multiple shoots at different concentrations of cytokinins kinetin, BA, zeatin and TDZ. Values are the means of three replicates with 50 explants in each replication. Error bars represent the standard error from the mean.

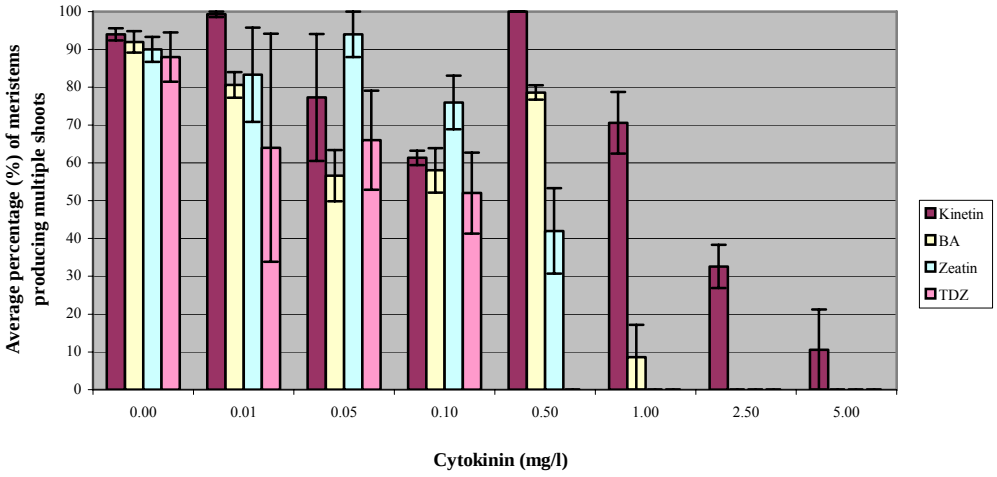

Figure 5.4: Average percentage of Palala shoot apical meristems producing multiple shoots at different concentrations of cytokinins kinetin, BA, zeatin and TDZ. Values are the means of three replicates with 50 explants in each replication. Error bars represent the standard error from the mean.

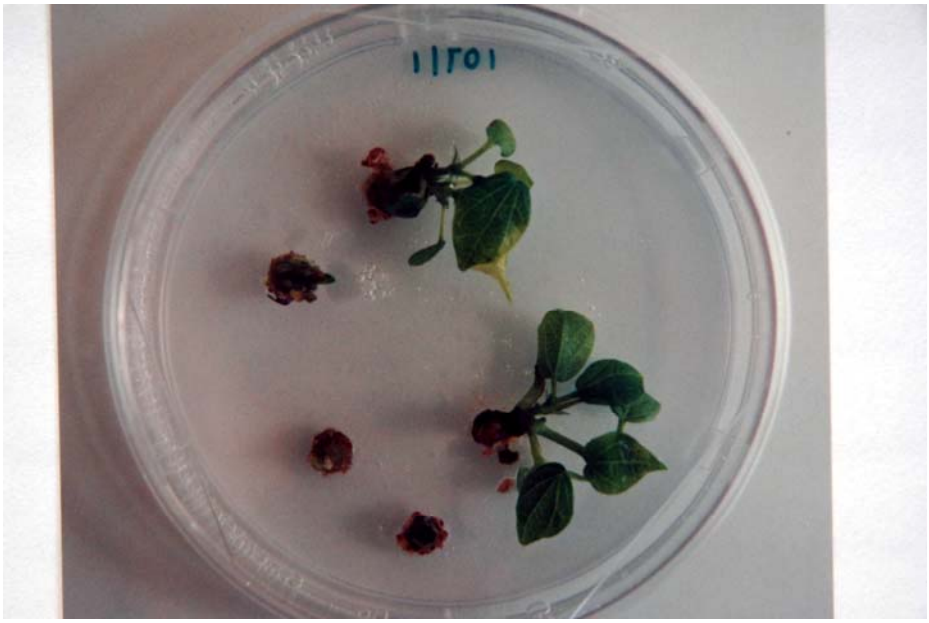

Figure 5.5: Shoot development from 107/1 shoot apical meristems after nine days on MS medium (*bar* 10 mm).

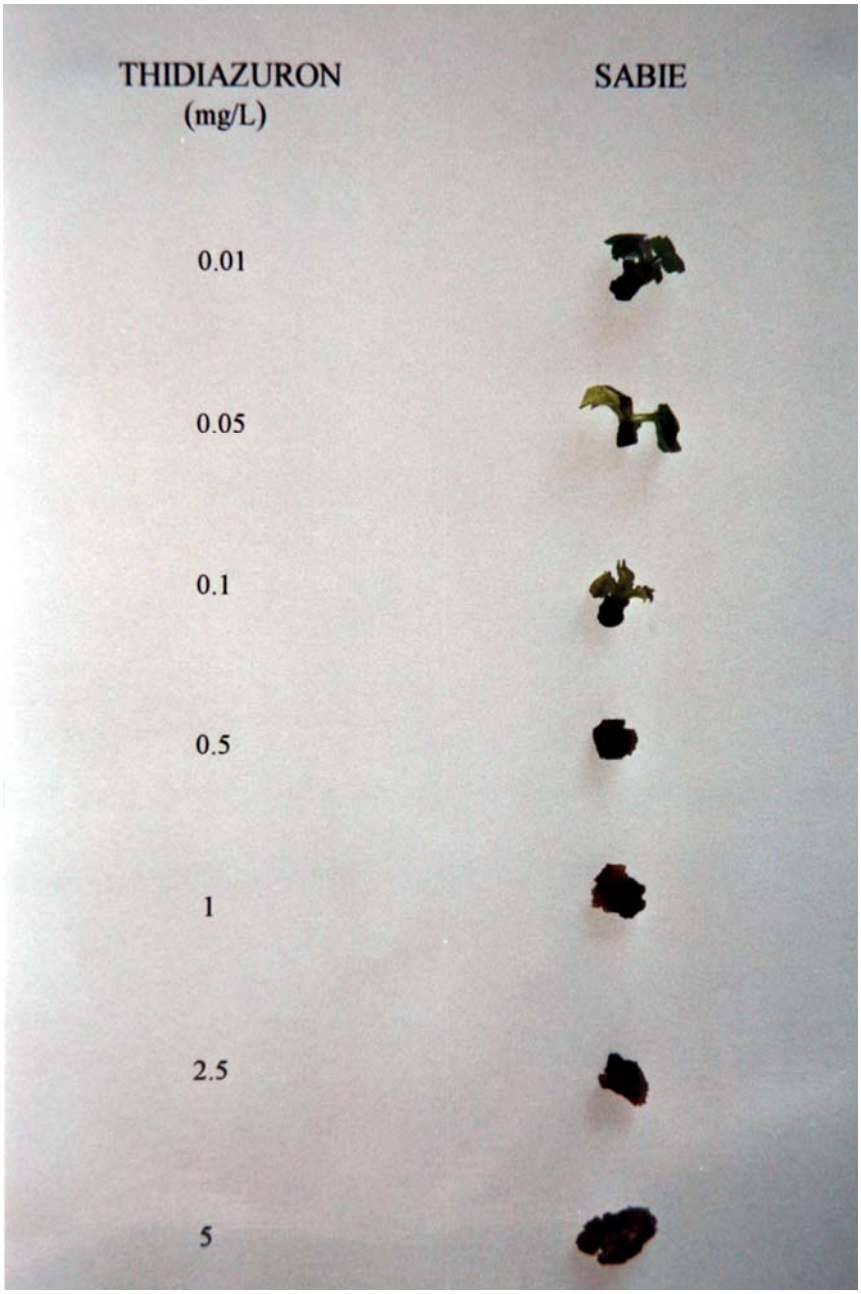

Figure 5.6: Shoots (s) and callus (c) formation from shoot apical meristems of Sabie on various concentrations of TDZ (*bar* 10 mm).

or more TDZ caused loss of apex organization and promoted callusing of the shoot meristems for cultivars Sabie, 107/l and Palala. Media containing 1 mg/l or more TDZ caused loss of apex organization and promoted callusing of the shoot meristems of cultivar LRCC 101.

Shoot meristems from all four cultivars differentiated into multiple green shoots on all concentrations of kinetin tested (except for LRCC 101 on medium containing 5 mg/l) after four weeks in culture. The highest percentage of shoot apical meristems producing multiple shoots was at different concentrations of kinetin for the different cultivars: 0.1 mg/l for Sabie (100%), 0.05 and 1 mg/l for LRCC 101 (93.3%), 1 mg/l for 107/1 (99.3%) and 0.5 mg/l for Palala (100%) (Figs. 5.1, 5.2, 5.3 and 5.4). In Figure 5.7 results of Sabie shoot apical meristems producing shoots on 0.1 mg/l kinetin after four weeks are shown. The number of shoots produced per shoot apical meristem explant varied among the four cultivars: 5-6 for Sabie and Palala, 4-5 for 107/1 and 3-4 for LRCC 101. At concentrations higher or lower than the concentration on which the most shoot apical meristems produced multiple shoots, the percentage of shoot apical meristems producing shoots was reduced (Figs. 5.1, 5.2, 5.3 and 5.4).

Shoot meristems from all four cultivars differentiated into multiple green shoots when 0.01-0.5 mg/l zeatin was added to the medium (Figs 5.8 and 5.9). The highest percentage of shoot apical meristems producing shoots was at different concentrations of zeatin for the different cultivars: 0.01 mg/l for Sabie (95.3%), 0.05 mg/l for LRCC 101 (99.3%), 0.01 mg/l for 107/1 (99.3%) and 0.05 mg/l for

Figure 5.7: Sabie shoot apical meristems producing shoots after four weeks on MS medium containing 0.1 mg/l kinetin (*bar* 10 mm).

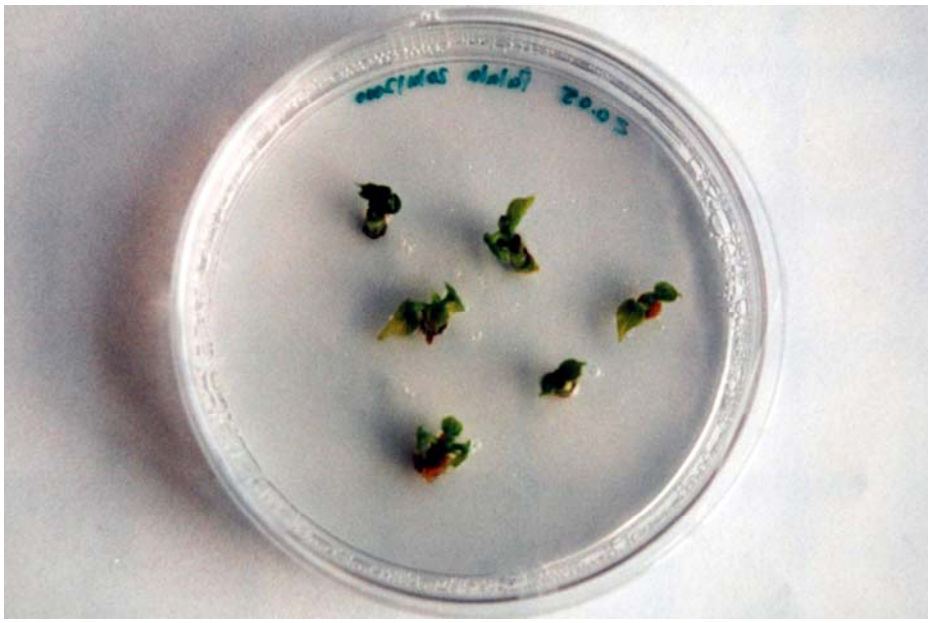

Figure 5.8: Palala shoot apical meristems producing shoots on MS medium containing 0.05 mg/l zeatin (*bar* 10 mm).

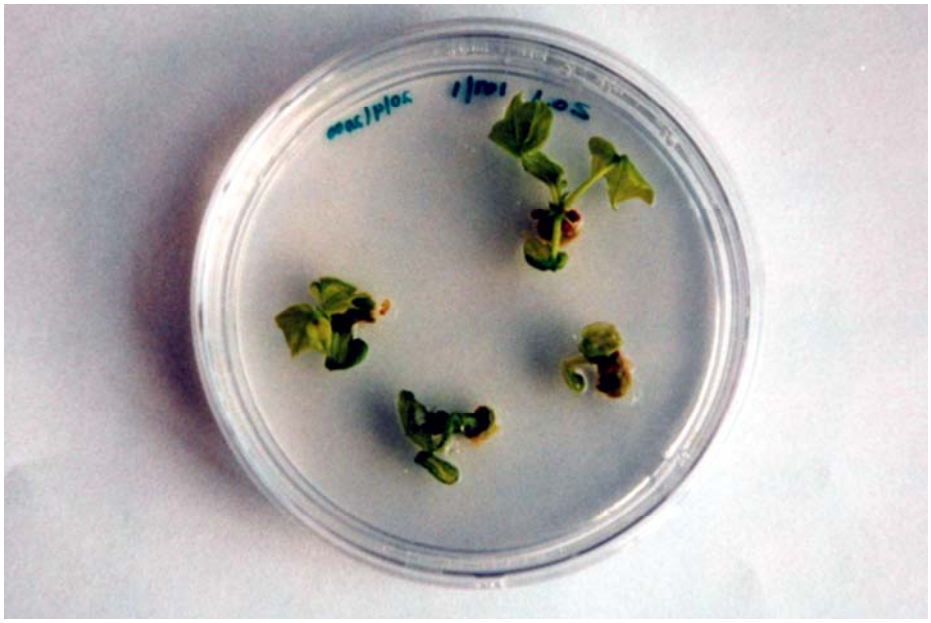

Figure 5.9: 107/1 shoot apical meristems producing shoots on MS medium containing 0.1 mg/l zeatin (*bar* 10 mm).

Figure 5.10: Sabie shoot apical meristems producing shoots on MS medium containing 0.1 mg/l BA (*bar* 10 mm).

Palala (94%) (Figs. 5.1, 5.2, 5.3 and 5.4). The number of shoots produced per shoot apical meristem explant varied among the four cultivars: 3-5 for Sabie, 107/1 and LRCC 101 and 3-4 for Palala. For zeatin concentrations higher than 0.5 mg/l for all cultivars, the explants died and browning of the medium around the explant inserted in the medium occurred.

Shoot meristems from Sabie and LRCC 101 differentiated into multiple green shoots when 0.01-0.5 mg/l BA was added to the medium. Shoot meristems from 107/1 and Palala differentiated into multiple green shoots when 0.01-1 mg/l BA was added to the medium. The highest percentage of shoot apical meristems producing multiple shoots was at different concentrations of BA for the different cultivars: 0.05 mg/l for Sabie (85.3%), 0.05 mg/l for LRCC 101 (82%), 0.1 mg/l for 107/1 (66%) and 0.01 mg/l for Palala (80.6%) (Figs. 5.1, 5.2, 5.3 and 5.4). The number of shoots produced per meristem explant was 3-5 for Sabie and 107/1, 3-4 for Palala and LRCC 101. For BA concentrations higher than 0.5 and 1.0 mg/l, the meristem explants died and browning of the medium around the explant inserted in the medium occurred.

The results of the logistic regression are shown in Table 5.1. From the results it was evident that the type of cytokinin tested and the concentration of that cytokinin had a significant effect on the production of multiple shoots from the shoot apical meristems of Sabie, 107/1, LRCC 101 and Palala. The type of cultivar also had a significant effect on the production of multiple shoots from the

Table 5.1: Analysis of effects from the logistic regression for Sabie, 107/1, LRCC 101 and Palala.

	Type 3 Analysis of Effects		
Effect	DF	Wald Chi-Square	Pr>ChiSq
Concentration	1	1664.8645	<0.0001
Cytokinin	3	526.0164	<0.0001
Cultivar	3	169.7816	<0.0001
Concentration*Cultivar	3	192.9027	<0.0001
Concentration*Cytokinin	3	1376.9060	<0.0001
Cultivar*Cytokinin	9	192.9027	<0.0001

If Pr>ChiSq <0.05, the effect is considered significant

shoot apical meristems at different concentrations of the cytokinin and also at the different cytokinins tested.

The presence of kinetin (0.01-2.5 mg/l) in the regeneration medium induced multiple shoots from the shoot apical meristems of the four cultivars (Figs. 5.1, 5.2, 5.3 and 5.4). The percentage (100%) of Sabie shoot apical meristems producing multiple shoots on medium containing 0.1 mg/l kinetin was considerably higher than the percentage (42%) of meristem explants producing multiple shoots on MS medium with 5.0 mg/l kinetin. The percentage (93.6%) of LRCC 101 shoot apical meristems producing multiple shoots on medium containing 0.05 and 1.0 mg/l kinetin was appreciably higher than the percentage (10.7%) of meristem explants on MS medium with 2.5 mg/l kinetin.

The percentages (93.3% and 99.3%) of 107/1 shoot meristems producing multiple shoots on medium containing 0.01 mg/l and 1 mg/l kinetin, respectively, were higher than the percentage (48.6%) of meristem explants on MS medium with 2.5 mg/l kinetin. The percentages (99.3% and 100%) of Palala shoot apical meristems producing multiple shoots on medium containing 0.01 mg/l and 0.5 mg/l kinetin, respectively, were appreciably higher than the percentage (10.6%) of meristem explants on MS medium with 5.0 mg/l kinetin.

At the concentration of 0.1 mg/l kinetin, the percentages (100% and 91.3%) of shoot apical meristems of Sabie and LRCC 101, respectively, producing multiple shoots were higher than the percentages (68.6% and 61.3%) of shoot apical

meristems of 107/1 and Palala, respectively, producing multiple shoots. At 0.5 mg/l kinetin the percentages (90%, 88.7% and 100%) of shoot apical meristems of Sabie, LRCC 101 and Palala, respectively, were higher than the percentage (54%) of 107/1 shoot apical meristems producing multiple shoots.

For Sabie, the addition of 0.01, 0.1 and 0.5 mg/l BA to the medium resulted in lower percentages (66.6%, 62% and 80%, respectively) of shoot apical meristems producing multiple shoots, when compared to the higher percentage (90.6%) of meristems producing multiple shoots when no BA was added to the medium, indicating that BA did not enhance percentage of Sabie shoot meristems producing multiple shoots. For cultivar LRCC 101, it was evident that the percentages (60.3%, 78.7% and 56.7%) of shoot apical meristems producing mltiple shoots were lower when 0.01, 0.10 and 0.5 mg/l BA, respectively, was added to the medium when compared with the percentage (88.6%) of meristem explants producing multiple shoots on MS medium without BA. For cultivar 107/1, the addition of 0.01-1 mg/l BA to the medium resulted in lower percentages (65.3%, 58%, 66%, 16.7% and 8.5%, respectively) of shoot apical meristems producing multiple shoots when compared with the percentage (80%) of meristem explants producing multiple shoots when no BA was added to the medium. For cultivar Palala, the addition of 0.01-1 mg/l BA to the medium resulted in lower percentages (80.6%, 56.6%, 58%, 78.6% and 8.6%, respectively) of shoot apical meristems producing multiple shoots when compared with the percentage (92%) of meristem explants producing multiple shoots when no BA was added to the medium.

When 0.05 mg/l BA was added to the medium, the percentages (58% and 56.6%) of shoot apical meristems of 107/1 and Palala, respectively, producing multiple shoots were considerably lower than the percentages (85.3% and 82%) of Sabie and LRCC 101 shoot apical meristems, respectively, producing multiple shoots. When 0.5 mg/l BA was added to the medium, the percentages (80% and 78.6%) of shoot apical meristems of Sabie and Palala, respectively, producing multiple shoots were higher than the percentages (56.7% and 16.7%) of LRCC 101 and 107/1 shoot apical meristems, respectively, producing multiple shoots.

The percentage (95.3%) of Sabie shoot apical meristems producing multiple shoots on medium containing 0.01 mg/l zeatin was higher than the percentage (84%) of shoot apical meristems producing multiple shoots on medium without zeatin. The percentages (93.3% and 99.3%) of LRCC 101 shoot apical meristems, respectively, producing multiple shoots on medium containing 0.01 and 0.05 mg/l zeatin were appreciably higher than the percentage (48.7%) of meristem explants producing multiple shoots on medium containing 0.5 mg/l zeatin. The percentages (99.3% and 98%) of 107/1 shoot apical meristems producing multiple shoots on medium containing 0.01 and 0.1 mg/l zeatin, respectively, were appreciably higher than the percentage (22%) of meristem explants producing multiple shoots on medium containing 0.5 mg/l zeatin. The percentages (83.3% and 94%) of Palala shoot apical meristems producing multiple shoots on medium containing 0.01 and 0.05 mg/l zeatin, respectively, were appreciably higher than the percentage (42%) of meristem explants producing multiple shoots on medium containing 0.5 mg/l zeatin.

When 0.5 mg/l zeatin was added to the medium, the percentage (78%) of shoot apical meristems of Sabie producing multiple shoots was appreciably higher than the percentages (48.7%, 22% and 42%) of LRCC 101, 107/1 and Palala shoot apical meristems, respectively, producing multiple shoots.

When TDZ was added to medium at concentrations of 0.05 and 0.1 mg/l, there was a reduction in the percentages of shoot apical meristems producing multiple shoots from Sabie, LRCC 101, 107/1 and Palala when compared to the percentages (95.3%, 96%, 87.3% and 88%, respectively) of shoot apical meristems producing multiple shoots when no TDZ was added to the medium. The percentage (80.6%) of Sabie shoot apical meristems producing multiple shoots on medium containing 0.01 mg/l TDZ was considerably higher than the percentages (46%, 46.7% and 64%) of LRCC 101, 107/1 and Palala shoot apical meristems, respectively, producing multiple shoots on the same medium.

One hundred healthy green shoots (2-3 cm) for each cultivar were excised from the shoot apical meristems cultured on kinetin at the concentrations which gave the highest percentage of meristems producing multiple shoots: 0.1 mg/l for Sabie (100%), 0.05 mg/l for LRCC 101 (93.3%), 1.0 mg/l for 107 and 0.5 mg/l for Palala and transferred to MS medium containing 0.1 mg/l kinetin before transfer to rooting medium. All one hundred shoots for each cultivar elongated to a height of 3-5 cm.

5.3.2 Rooting of *in vitro* shoots

Root initiation from all 16 shoots of the four cultivars did not occur on half-strength MS medium with or without 0.05 mg/l and 0.1 mg/l NAA or IBA. This hormone treatment resulted in tissue mortality. The percentage of shoots which rooted on full strength MS medium containing 0.1 mg/l kinetin varied among the cultivars: 90% (18/20) for Sabie, 70% (14/20) for Palala and 107/1 and 80% (16/20) for LRCC 101 (Table 5.2). Rooting was maintained on this medium for four weeks. All the shoots elongated and a well developed rooting system developed (Figs. 5.11 and 5.12). These plantlets were transferred to sterile potting mixture and placed in the growth chamber for further growth (Fig. 5.13). These plantlets grew and after four months, they bore flowers (Fig. 5.14) and bolls (Fig. 5.15) and produced viable seed under growth chamber conditions. The overall frequency of mature regenerated cotton plants per meristem was 90% for Sabie, 74.6% for LRCC 101, 69.5% for 107/1 and 70% for Palala (Table 5.2). All regenerated plants that were advanced to soil were phenotypically (height of plant; shape, width and length of leaves; size, shape and colour of flowers, size of bolls) normal and appeared morphologically similar to seed-derived cotton plants (Fig. 5.16).

Figure 5.11: Sabie shoot rooting on MS medium containing 0.1 mg/l kinetin (*bar* 10 mm).

Figure 5.12: Rooting of a LRCC 101 shoot on MS medium containing 0.1 mg/l kinetin (*bar* 10 mm).

Figure 5.13: Sabie plant (left) and 107/1 plant (right) in sterile potting mixture (*bar* 10mm).

Figure 5.14: Sabie regenerated plant bearing flowers.

Figure 5.15: Sabie regenerated plant showing boll production.

Table 5.2: Data on percentage of shoot apical meristems (from the initial 150) producing multiple shoots on medium containing kinetin, percentage of rooted shoots on MS containing 0.1 mg/l kinetin and frequency of mature regenerated plants per meristem for cotton cultivars Sabie, LRCC 101, 107/1 and Palala.

Cultivar	% meristems producing shoots	% rooted shoots	% regenerated plants/meristem
Sabie	100	90 (18/20)	90
LRCC 101	93.3	80 (16/20)	74.6
107/1	99.3	70 (14/20)	69.5
Palala	100	70 (14/20)	70

Figure 5.16: Regenerated 107/1 (middle) and Sabie (right) plants in potting mixture. All regenerated 107/1 plants were phenotypically normal and appeared morphologically similar to the seed derived 107/1 plant (left), (*bar* 10 mm).

5.4 Discussion

The cultivation of transgenic cotton rapidly gained significant ground in the late 1990s, and now accounts for the majority of cotton in production in the U.S. and many other countries (Mishra *et al*., 2003). Yet, despite the commercial success of genetically modified cotton, the transformation and regeneration of cotton via somatic embryogenesis is not a trival process by any means, and cotton remains one of the more recalcitrant species to manipulate in culture (reviewed in Wilkins *et al*., 2000). Embryogenic potential is a polygenic, low heritable trait (Kumar *et al*., 1998) that is highly genotype dependent (Trolinder and Xhixian, 1989; Nobre *et al*., 2001; Rauf and Rahman, 2005).

Efforts to surmount the genotype dependent barrier using *Agrobacterium*-mediated transformation (Zapata *et al*., 1999a; Satyavathi *et al*., 2002) or particle bombardment (McCabe and Martinell, 1993) of meristems have been met with some success. Protocols for the regeneration of plants from shoot apical meristems of cotton have been reported (Zapata *et al*., 1999b; Satyavathi *et al*., 2002) but not for the South African commercially grown cotton cultivars Sabie, 107/1, Palala and LRCC 101. This study was undertaken to develop regenerated cotton plants from these commercially grown South Africa cotton cultivars. The underlying premise being that genotype independent transformation and regeneration allows for the introduction of transgenes directly into elite genetic backgrounds, and satisfies increasing demands for the rapid delivery of improved cultivars into commercial production (Mishra *et al*., 2003). This study was

successful in this regard, in that regenerable Sabie, Palala, 107/1 and LRCC 101 cultivars have been developed. These regenerable cotton cultivars promise to increase efficiency of developing transgenic cotton plants while simultaneously decreasing production costs, and bear significant ramifications for improvement strategies in molecular breeding programmes (Mishra *et al*., 2003).

In this study, the effects of cytokinins BA, kinetin, zeatin and TDZ on the production of shoots from the shoot apical meristems of Sabie, 107/1, LRCC 101 and Palala was investigated. Cytokinins are known to induce axillary as well as adventitious shoot formation from meristematic explants (Sujatha and Reddy, 1998).

In this study, MS medium induced multiple shoots from the shoot apical meristem at a frequency of 84-95.3% for Sabie, 88.6-96% for LRCC 101, 80-95.3% for 107/1 and 88-94% for Palala. These results are consistent with the results reported by Gould *et al*. (1991a) that 83-100% of shoot apices of cotton (cultivars Acala SJ-2; Coker 310, 312; DeltaPine 50, 90; Paymaster 145, 404; Stoneville 213, 452, 506) produced shoots when placed on MS medium. In their study, when tissues were re-cultured onto this media at bi-weekly intervals, shoots progressed rapidly to the four leaf stage within five to six weeks. Zapata *et al*. (1999b) reported that in their study, the highest percentage (58%) of regenerated cotton (cultivars Tamcot Sphinx, MAR-CUBQHRPIS and Coker 312) plants from the shoot apex was obtained in full-strength MS plus vitamins without any growth regulators. Similarly, Saeed *et al*. (1997) reported that MS simple medium was

suitable for shoot tip growth in 19 cotton cultivars. Hemphill *et al*. (1998) reported that shoot meristems of cotton cultivars, Paymaster HS26, CA-3076, Stoneville 7A and Stoneville 474, placed on MS medium produced shoots, however, failed to yield elongated shoots (2-3 cm or greater) during the three week culture period.

The percentage of shoot apical meristems producing multiple shoots was influenced by the type and concentration of the cytokinin. In the present study, MS medium supplemented with BA (0.01-0.5 mg/l) induced multiple shoots from the shoot apical meristems of all four cultivars (3-4/meristem for Palala and LRCC 101 and 3-5/meristem for Sabie and 107/1). Similarly, Hemphill *et al*. (1998) reported that apices isolated from *in vitro* grown 1-day-old and 28-day-old seedlings (Stoneville 7A and Paymaster HS26) formed shoots when cultured on MS medium containing a range of BA levels (0.3, 0.5, 1.0 and 10 μm). In contrast, Satyavathi *et al*. (2002) reported that BA (0.05-0.3 mg/l) added to regeneration medium produced only single shoots from shoot apical meristems of the cotton cultivar MCU5. In the present study, concentrations of BA higher than 0.5 mg/l, i.e. 1, 2.5 and 5 mg/l did not induce shoots from cultivars Sabie and LRCC 101. These results are consistent with the results reported by Zapata *et al*. (1999b) and Hemphill *et al*. (1998) that shoot regeneration from the shoot apical meristem of various cotton cultivars was suppressed at the high concentration of 1 mg/l and 3.0 μm BA, respectively. Other researchers (Zapata *et al*., 1999b) have reported the failure of BA to stimulate multiple shoots from the shoot apex of kenaf. High concentrations of BA resulted in callus formation from the shoot

apical meristem of kenaf. In contrast, it has been reported that BA has been shown to be an effective cytokinin for shoot organogenesis from shoot apical meristems of other plant species, including soybean (Sato *et al*., 1993; Chapter 3 of this study), castor (Sujatha and Reddy, 1998) and sorghum (Zhong *et al*., 1998).

In the present study, the percentage of shoot apical meristems of Sabie and LRCC 101 producing multiple shoots on medium containing 0.5 mg/l BA, for example, was 80% and 56.7%, respectively, and for 107/1 and Palala, 16.7% and 78.6%, respectively. Statistically, it was shown that cultivar type did have a significant effect on the production of shoots from the shoot apical meristems at the different concentrations of cytokinins. This indicates that the shoot regeneration system described in this study is possibly genotype dependent at different concentrations of BA.

The presence of kinetin (0.01-5 mg/l) in the regeneration medium induced multiple shoots from the shoot apical meristems of the four cultivars (5-6/meristem for Palala and Sabie and 3-4/meristem for LRCC 101 and 4-5/meristem for 107/1). Similarly, Gould *et al*. (1991a) reported that medium containing 0.05-0.5 mg/l kinetin and various concentrations of IAA, supported normal shoot development from the shoot apices of various cotton cultivars (cultivars Acala SJ-2; Coker 310, 312; DeltaPine 50, 90; Paymaster 145, 404; Stoneville 213, 452, 506) with the highest percentage (100%) of shoot apices forming shoots when only 0.1 mg/l kinetin was added to the medium. Similarly,

in the present study, 100 % Sabie shoot apical meristems produced multiple shoots when 0.1 mg/l kinetin was added to MS medium. The addition of 0.1 mg/l kinetin to the medium enhanced the percentage of Sabie shoot apical meristems producing multiple shoots compared with the percentage (88%) of Sabie shoot apical meristems producing multiple shoots on medium containing no kinetin. Similarly, for 107/1, 0.01 and 1 mg/l kinetin enhanced the percentage of shoot apical meristems producing multiple shoots and for Palala, 0.01 and 0.5 mg/l kinetin enhanced the percentage of shoot apical meristems producing multiple shoots.

The effects of the media in the study by Gould *et al*. (1991a) did not become apparent until three to four weeks of culture. The difference in times at which the media took effect in this study (within two weeks) and the one by Gould *et al*. (1991a) could be due to the fact that the medium Gould and co-workers used contained kinetin and IAA and also to genotype differences. Saeed *et al*. (1997) reported that the best shoot development from meristem shoot tips was observed from MS medium containing 0.46 mM kinetin and that levels of kinetin higher than 2.32 mM did not show a good shoot response from shoot tips of cotton. It has been reported that kinetin regulates cell division and stimulates axillary and adventitious shoot proliferation when added to tissue culture medium (Saeed *et al*., 1997).

All cotton cultivars in this study produced shoots from the shoot apical meristems containing varying concentrations of kinetin. At higher concentrations (0.1, 2.5

and 5 mg/l) of kinetin, the percentage of shoot apical meristems producing multiple shoots was different for each cultivar, indicating that at higher concentrations of kinetin, shoot regeneration is possibly genotype dependent.

For the four test cultivars, media containing TDZ was not adequate for normal shoot development. Media containing 0.5 mg/l or more TDZ caused loss of apex organization and promoted callusing of the shoot meristem for cultivars Sabie, 107/l and Palala. The callus production was, in general, accompanied by the production of phenolic compounds. The shoots that were produced from the explants (2-3 per meristem for Palala and 107/1 and 2 per meristem for Sabie and LRCC 101) after four weeks at the lower concentrations of TDZ were small (>0.5 cm), did not elongate and eventually showed signs of chlorosis and became necrotic. For all four cultivars, the addition of 0.05 and 0.1 mg/l TDZ reduced the percentages of shoot apical meristems producing multiple shoots when compared with the percentages of shoot apical meristems producing shoots when no TDZ was added to the medium. TDZ was therefore concluded to be an ineffective cytokinin for shoot organogenesis from the shoot meristem of the four South African cotton cultivars tested. Similarly, Satyavathi *et al*., (2002) reported that TDZ resulted in the formation of callus from the cut ends of the shoot apical meristems of cotton cultivar MCU5 and a resulted delay in shoot response. Only single shoots were induced at the concentrations tested (0.05-0.3 mg/l). Other researchers have reported that TDZ at higher concentrations caused kenaf shoots to produce callus that exhibited vitrification (Srivatanankul *et al*., 2000).

This is the first report on the effects of various concentrations of zeatin on the production of shoots from shoot apical meristems of cotton. High percent shoot apical meristem response (76-99.3%) was achieved for all cultivars cultured on medium containing 0.01, 0.05 and 0.10 mg/l zeatin. When 0.5 mg/l zeatin was added to the medium, the percentage of shoot apical meristems producing multiple shoots was different for each cultivar, indicating that at this high concentration of zeatin, shoot regeneration is possibly genotype dependent.

In the present study, it was shown statistically that cultivar type did have a significant effect on the production of shoots from the shoot apical meristems at different concentrations of the cytokinins tested. This indicates that the shoot regeneration system described in this study is possibly genotype dependent at different concentrations of BAP, kinetin and zeatin. In contrast, other researchers have reported genotype independent shoot regeneration from shoot apical meristems. Zhong *et al*. (1998) reported on a genotype independent regeneration protocol from shoot apices of sorghum. Although the highest regeneration frequency in the genotypes tested in their study varied from 65% to 99% depending on the culture medium and genotypes, all 18 genotypes tested responded to multiplication of shoot apices at a relatively high efficiency on different combinations of 2,4-D and BA. Satyavathi *et al*. (2002) also reported that genotype seemed to have not much effect on shoot induction and shoot proliferation from the shoot apical meristems of three Indian cotton cultivars MCU5, DCH32, Coker 310FR. This was evident from the similar response of all varieties on different hormonal concentrations studied. Gould *et al*. (1991a) also

reported genotype independent regeneration from shoot apex tissues of cotton (cultivars Acala SJ-2; Coker 310, 312; DeltaPine 50, 90; Paymaster 145, 404; Stoneville 213, 452, 506). In future studies, more cotton cultivars need to be tested to determine whether the regeneration protocol in this study is genotype dependent.

The effectiveness of tissue-culture regimes is dependent on many factors including plant species, tissue type, size of explant, chemical properties and concentrations of cytokinins and auxins (Bowen, 1993).

In the present study, subculturing of the shoot apical meristem explant to fresh medium every two weeks was beneficial in that shoot proliferation was maintained. It has been reported that the best reculture media are those that do not deviate substantially from the initial culture medium (Gould *et al*., 1991a).

The induction of multiple shoots in explants varies with cytokinin type and concentration and is also influenced by the age of the cotton seedlings (Agrawal *et al*., 1997; Saeed *et al*. 1997; Satyavathi *et al*., 2002). In the present study, 5-7 day old seedlings were used and successful shoot regeneration occurred from the shoot apical meristems. In contrast, Saeed *et al*. (1997) reported that explant seedlings less than 2-days and more than 5-days old did not develop a good type of meristem. They found that it was difficult to excise and detect the meristem dome from the tips of less than two days. Tissue older than 4-5 days developed a small stem beyond the base of the cotyledons. At this stage, the tissue became smaller

in size and more watery with lots of hairs thus making the excision of the tip a tedious exercise. Only the explants between the age of 3-5 days were best for the excision of meristem tips in their study. Gould *et al*. (1991a) used apices from 5 day old cotton seedlings.

Saeed *et al*. (1997) reported that size of the meristem/shoot tip contributed significantly to the rate of plant formation. Mortality rate was highest (50%) when cotton meristems of less than 0.5 mm were cultured. In the present study, the size of the shoot apical meristem of all four cultivars used was 0.5-1 mm and high percentages (100%) of shoot apical meristems producing multiple shoots were obtained on medium containing 0.1 mg/l and 0.5 mg/l kinetin, for example. Similarly, Gould *et al*. (1991a) reported using cotton shoot apical meristems 0.5 mm in size. They reported that 85-100% shoot apical meristems produced shoots on medium containing 0.1 mg/l kinetin.

Shoots which were excised from the shoot apical meristems elongated on full strength MS medium containing 0.1 mg/l kinetin. Shoot elongation is essential before transfer to rooting media. The concentrations (0.05 mg/l and 0.1 mg/l) of NAA and IBA tested in half-strength MS did not induce rooting. Half-strength MS medium without growth regulators did not induce rooting either. These results are similar to the results reported by Saeed *et al*. (1997) that no root induction occurred from cotton shoots placed on half-strength MS medium. Gould *et al*. (1991a) reported that the concentration of 1, 2, 3, and 5 mg/l NAA failed to induce *in vitro* root formation from cotton shoots. The results obtained

in the present study, however, differ from the results reported by Saeed *et al*. (1997) that MS medium with 2.46 mM IBA did induce rooting from cotton shoots, and Hemphill *et al*. (1998) that cotton shoots rooted in MS medium containing 1µm/l IBA. Satyavathi *et al*. (2002) also reported that elongated cotton shoots were rooted on half strength MS medium containing 0.3 mg/l IBA. In the present study, shoots from all four cultivars cultured on full strength MS medium containing 0.1 mg/l kinetin produced roots (90% for Sabie, 70% for Palala and 107/1 and 80% for LRCC 101).

Theoretically, each excised shoot apex will develop into a rooted plant, however the yield of plantlets from isolated shoot apices depends on rooting efficiency. In the present study the overall frequency of regenerated plants per meristem was 90% for Sabie, 74.6% for LRCC 101, 69.5% for 107/1 and 70% for Palala. Rooting in cotton is genotype dependent and highly variable (10-93%) (Gould *et al*., 1991a; Hemphill *et al*., 1998). Gould *et al*. (1991a) reported that overall recovery of rooted shoots was 5-7% for cotton (Gould *et al*., 1991a). *In vitro* grafting of cotton shoots to seedling rootstock proved to be a genotype independent, simple and reliable method allowing 90-100% recovery of non-rooting shoots from culture (Luo and Gould, 1999). In situations where the shoot tissue is valuable, i.e., transgenic shoots regenerating in culture, inefficient rooting produces a significant loss (Luo and Gould, 1999).

Shoot apex culture has been used successfully with many monocot and dicot families (Murashige, 1974; Gould *et al*., 1991; Sato *et al*., 1993; Hemphill *et al*.,

1998; Zhong *et al*., 1998; Satyavathi *et al*., 2002; Matsuda *et al*., 2005). Theoretically, the tissues of the apical meristem are best suited for use in plant propagation and regeneration because these tissues are programmed for shoot organogenesis and do not need to differentiate to a meristematic state (Gould *et al*., 1991a). The advantages of shoot meristem culture over other regeneration systems are many fold. Shoot regeneration from shoot apical meristem is direct, relatively simple and needs less time to regenerate large numbers of plants (Saeed *et al*., 1997). Plants regenerated from shoot meristems are true to phenotype with low incidence of somaclonal variation and chromosomal abnormalities (Murashige, 1974; Bajaj, 1998). Shoot meristem explant has few genotype limitations and is considered as more appropriate because meristematic cells are programmed for direct shoot organogenesis without an intervening callus stage (Zapata *et al*., 1999b).

All the plants regenerated from the four commercially grown South African cotton cultivars exhibited normal phenotype, flowered and set seed. These results are similar to the results reported by Gould *et al*. (1991a), Saeed *et al*. (1997), Hemphill *et al*. (1998), Zapata *et al*. (1999b) and Satyavathi *et al*. (2002). Normal phenotype should be expected of plants regenerated from shoot apical meristems (Murashige, 1974; Bajaj, 1998). In comparison, cotton plants regenerated by somatic embryogenesis produce many abnormal phenotypes and cytogenetic aberrant plants (Kumria *et al*., 2003).

In future studies, the effect of combinations of cytokinins used in this study on the percentage of shoot apical meristems producing multiple shoots from Sabie, LRCC 101, Palala and 107/1 could be assessed, as well as combinations with various auxins. Other South African commercially grown cotton cultivars should be evaluated in the future to determine whether the regeneration protocol described in this study is genotype dependent. Future work on rooting of elongated shoots could include a wider range of concentrations of NAA and IBA as well as different environmental conditions (e.g. light) and medium compositions (e.g. addition of calcium and magnesium).

5.5 Conclusions

The type of cytokinin tested and the concentration of that cytokinin had a significant effect on the production of multiple shoots from the shoot apical meristems of Sabie, 107/1, LRCC 101 and Palala. The type of cultivar also had a significant effect on the production of multiple shoots from the shoot apical meristems at different concentrations of the cytokinin and also at the different cytokinins tested.

The highest percentage (90-100%) of shoot apical meristems of all four cultivars producing multiple shoots was on medium containing either kinetin or zeatin. The concentrations of kinetin which produced the highest percentage (100%, 93.3%, 99.3% and 100%) of shoot apical meristems of Sabie, LRCC 101, 107/1 and Palala producing multiple shoots, were 0.1 mg/l, 0.05 and 1.0 mg/l, 1 mg/l

and 0.5 mg/l, respectively. The concentrations of zeatin which produced the highest percentage (95.3%, 99.3%, 99.3% and 94%) of shoot apical meristems of Sabie, LRCC 101, 107/1 and Palala producing multiple shoots, were 0.01 mg/l, 0.05 mg/l, 0.01 mg/l and 0.05 mg/l, respectively. Shoot regeneration from shoot apical meristems appeared to be genotype dependent at the different concentrations of cytokinins. The recovery of rooted shoots from the initial isolated 20 shoots for each cultivar in this study was 90% for Sabie, 70% for Palala and 107/1 and 80% for LRCC 101. The overall frequency of regenerated plants per meristem was 90% for Sabie, 74.6% for LRCC 101, 69.5% for 107/1 and 70% for Palala. The establishment of a rapid, efficient, simple and possibly genotype dependent plant regeneration system from the shoot apical meristems of the commercially grown cotton cultivars Sabie, 107/1, Palala and LRCC 101, is described. The results obtained in this study are potentially promising, since data on the regeneration of the commercially grown South African cotton cultivars Sabie, LRCC 101, Palala and 107/1 are not available. This is the first report of a successful regeneration protocol for these South African cotton cultivars. This shoot apical meristem-based regeneration method can be applied to plant transformation, either by particle bombardment or *Agrobacterium*-mediated gene transfer for the introduction of foreign genes, e.g. the *pat* gene for herbicide resistance, into commercially grown South African cotton cultivars. Of the plant genera regenerated *in vitro* from shoot apices, cotton has been the most difficult (Gould *et al*., 1991). Despite the recalcitrance of this genus, use of the shoot apical meristem can make a wider range of cotton germplasm accessible to improvement by the currently available transformation methods.

C H A P T E R 6

Transformation of the shoot apical meristems of commercially grown South African cotton cultivars with *Agrobacterium tumefaciens* containing the *pat* gene for resistance to the herbicide phosphinothricin.

Sections of this chapter have been presented as a paper at the World Cotton Research Conference-2, Divani-Caravel Hotel, Athens, Greece, 6-12 September 1998.

C. McNaughton and M.E.C. Rey. Development of an efficient regeneration and transformation system for commercially grown South African cotton cultivars.

Abstract

In search of establishing a system for genetic transformation of South African commercially grown cotton (*Gossypium hirsutum* cvs Sabie, Palala, LRCC 101 and 107/1) for improving cultivars, a method of *Agrobacterium*-mediated transformation of isolated shoot apical meristems of seedlings was developed to produce herbicide resistant plants. Prior to transformation experiments, the concentrations of the antibiotics, cefotaxime and carbenicillin, to effectively eliminate *Agrobacterium* but not affect shoot regeneration, were determined. These were found to be 50 mg/l for cefotaxime and 400 mg/l for carbenicillin. The concentrations of the selection agents PPT and kanamycin in the selection medium, which would kill non-transformed shoot apical meristems, were also determined. The percentage of shoot apical meristems of all four cotton cultivars producing shoots after two-three weeks on medium containing 0.1 mg/l PPT were 91.6% for Sabie, 66% for 107/1, 72% for LRCC 101 and 53% for Palala. When the cotton cultivars were placed onto selection medium containing 25 mg/l kanamycin, 60% of Sabie, 66.6% of 107/1, 59% of LRCC 101 and 69% of Palala shoot apical meristems produced multiple shoots. Concentrations of 0.5-20 mg/l PPT and 50-150 mg/l kanamycin inhibited 100% shoot and root formation, and so 1 mg/l PPT and 100 mg/l kanamycin were chosen for selection of transformants. Prior to transformation experiments, the effect of wounding (with a needle) and injecting water and 20 μM acetosyringone on shoot regeneration from 20 shoot apical meristems of all four cotton cultivars isolated from 5-7 day old seedlings, was assessed. After four weeks in culture, 100% shoot regeneration occurred

from wounded meristems of all four cotton cultivars indicating no damage to meristem tissue. One hundred shoot apical meristems (0.5-1 mm in length) isolated from 5-7 day old seedlings were wounded three times with a sterile needle and then transformed by injecting 10 µl of the 48 h *A. tumefaciens* strain LBA4404 (harbouring the recombinant vector pBI101 containing the *pat* gene and the marker gene *npt II*) into the shoot apical meristems. One hundred shoot apical meristems that were not wounded were placed into the 48 h transconjugate *A. tumefaciens* culture for 30 minutes and then blotted dry. After the transformed shoot apical meristems were co-cultivated with *Agrobacterium* for two days, they were transferred to regeneration medium containing 0.01 mg/l kinetin, 50 mg/l cefotaxime and 400 mg/l carbenicillin for one week. After a week they were placed onto regeneration medium containing 0.01 mg/l kinetin, 1 mg/l PPT and 100 mg/l kanamycin for selection of transformed shoots. One hundred percent non-transformed control explants turned brown and became necrotic within one week on the selection medium. After two weeks on selection medium, green healthy shoots were produced from 34% and 25% wounded Sabie and 107/1 shoot apical meristems, respectively. The percentage of non-wounded shoot apical meristems producing multiple shoots was 15% for Sabie and 12% for 107/1. Five-six shoots/meristem and 4-5 shoots/meristem were produced from wounded and non-wounded Sabie shoot apical meristems, respectively. Four-five shoots/meristem and 3-5 shoots/meristem were produced from wounded and non-wounded 107/1 shoot apical meristems, respectively. These results showed that the shoot apical meristems were transformed with the *pat* gene that was successfully detoxifying the herbicide PPT. One hundred percent shoot

meristems of Palala and LRCC 101 did not produce transformed shoots and became necrotic within two weeks. Green, putative transgenic shoots (3-5cm) of Sabie and 107/1 from wounded and non-wounded shoot apical meristems were placed onto medium containing 100 mg/l kanamycin and 1 mg/l PPT to induce rooting. Fifteen (from 187 shoots) and ten shoots (from 113 shoots) from wounded Sabie and 107/1 meristems, respectively, rooted and ten (from 68 shoots) and five shoots (from 48 shoots) from non-wounded Sabie and 107/1 meristems, respectively, rooted. The percentage of rooted shoots from wounded and non-wounded Sabie meristems was therefore 8% and 15%, respectively, and 9% and 8% for wounded and non-wounded 107/1 meristems, respectively. The frequency of transformed Sabie plants per meristem was 3% and 2.3% for wounded and non-wounded meristems, respectively. The frequency of transformed 107/1 plants per meristem was 2.3% and 1.2% for wounded and non-wounded meristems, respectively. The presence of the *pat* gene in various shoots of the transformed cotton plants was confirmed by PCR, indicating successful transformation of meristematic tissues and the presence of non-chimeric transgenic plants. All transgenic plants with healthy roots were transferred to sterile potting mixture in tissue culture flasks and then acclimatized in the growth chamber. All these transformed plantlets lost their leaves and died three weeks later.

6.1 Introduction

Cotton [*Gossypium* (*G*.) *hirsutum* L.] is a major fibre crop worldwide and its economic importance is increasing annually (Regier *et al*., 1986; Perlak *et al*., 1990; John, 1997; Ganesan and Jayabalan, 2004; Li *et al*., 2004). Cotton is of particular importance in the textile industry, with nearly 50 % of the total textile fibre consumption depending on cotton fibres (Peeters *et al*., 1994). Cotton is also cultivated for its essential seed oil (Ganesan and Jayabalan, 2004), the world's second most important oilseed crop after soybean (Nobre *et al*., 2001). In addition to textile manufacturing, cotton and cotton by-products provide raw materials that are used to produce a wealth of consumer-based products, foodstuffs, livestock feed, fertilizer and paper (Mishra *et al*., 2003).

Cotton is grown in more than 80 countries and has an annual production of 20 million tons (Li *et al*., 2004). Cotton production is of high economic importance in Africa. Cotton is grown on 2.5 million ha of the continent, most of it on small plots of less than 10 ha (Scott, 2004). In 2004-2005 South Africa cotton was grown on 36 303 ha of land in various regions by both commercial and small-scale farmers (http://www.cottonsa.org.za). Cotton production areas in South Africa are the Northern Cape-Lower Orange River and Griqualand West, North West-Rustenberg, Northern Province- Limpopo Valley, Loskop and Springbok Flats, Mpumalanga and KwaZulu-Natal (http://www.cottonsa.org.za).

A rich genetic reservoir is available in wild and wild relatives of cotton, but genetic improvement through interspecific hybridization is hampered by incompatibility barriers. There has long been interest in the genetic improvement of this valuable crop species (John, 1997). Cotton species are continually being improved by traditional (conventional) breeding techniques and a number of programmes have been developed aimed at improving existing genotypes using genetic engineering techniques (Llewellyn *et al*., 1990; Rajasekaran *et al*., 1996; Nobre *et al*., 2001; Mishra *et al*., 2003). Cotton biotechnology plays a vital role in improving the quality as well as the quantity of fibre by producing plants resistant to insect, herbicide, fungi, bacteria and nematodes through genetic engineering (Pannetier *et al*., 1997; Leelavathi *et al*., 2004). Improvement in yield and other qualities is expected to expand and enhance the marketability of cotton (Satyavathi *et al*., 2002). Cotton biotechnology hinges on two tightly interlaced processes- transformation and regeneration.

Agrobacterium provides one of the main vehicles for introducing foreign DNA into plants. *Agrobacterium*-mediated DNA transformation has some advantages over direct transformation methods in reducing the copy number of the transgene and potentially leading to fewer problems with transgene co-suppression and instability. Other advantages include defined transgene integration and fewer rearrangements within the inserts (Kohli *et al*., 1999). The use of this transformation system normally results in clonally transformed plants, in contrast to the large number of mosaic plants usually obtained by direct transformation

(Neuhuber *et al*., 1994; Bastar *et al*., 2004). One significant limitation of this method, however, is that transformation appears to be genotype dependent.

Regeneration of somatic embryos, used together with the callus-based transformation procedure developed by Horsch and collegues (1985), has been successfully used in *Agrobacterium*-mediated transformation of cotton (Firoozabady *et al*., 1987; Umbeck *et al*., 1987; Perlak *et al*., 1990; Bayley *et al*., 1992; Pannetier *et al*., 1997; Sacks *et al*., 1998; Kohel and Yu, 1999; Jin *et al*., 2005; Tohidfar *et al*., 2005) and in particle bombardment procedures (Finer and McMullen, 1990; Rajasekaran *et al*., 1996) to obtain insect, herbicide and disease resistant plants. Fertile, phenotypically normal transformed plants have been produced by these methods of transformation and foreign genes in cotton are inherited and expressed in a Mendelian fashion (Tohidfar *et al*., 2005). However, these advances presently remain restricted to the cultivars that can be regenerated from calli and cell suspensions by somatic embryogenesis. Mostly, only Coker varieties have been transformed and regenerated consistently (Trolinder and Goodin, 1987; Firoozabady *et al*., 1987; Umbeck *et al*., 1987; Rajasekaran *et al*., 1996; Satyavathi *et al*., 2002). There have also been reports on transformation of cotton by particle bombardment of embryogenic cell suspension cultures (Rajasekaran *et al*., 2000). However, transgenic plants from cell cultures older than six months produced plants with abnormal morphology and a high degree of sterility. Somaclonal effects due to mutations during tissue culture and regeneration processes and/or the site of gene insertion into the plant genome have been found to influence gene expression in regenerated cotton lines (Sacks *et al*.,

1998). Recently, Li *et al*. (2004) have reported a method for the genetic transformation of cotton pollen by means of vacuum infiltration and *Agrobacterium*-mediated T-DNA transfer. This transformation method can serve as an alternative to those demanding complex tissue culture techniques.

Agrobacterium-mediated transformation and regeneration of cotton via somatic embryogenesis remains the preferred method of choice for generating transgenic cotton. Published *Agrobacterium*-mediated methods require approximately 10-12 months, or longer, to regenerate transgenic cotton plants (Firoozabady *et al*., 1987; Umbeck *et al*., 1987; Trolinder and Goodin. 1987, 1988a and b), however, somaclonal variation becomes problematic with such long culturing times (Rajasekaran, 1996). More recently, however, a method of transforming embryogenic callus with *Agrobacterium* only took 3-5 months to produce 67 transgenic cotton plants (Leelavathi *et al*., 2004). Since the onset of blooming in regenerated plants varies considerably, seed production to produce genetically-stable transgenic lines requires an additional 6-8 months, meaning that each transgenic plant may take up to two years or more to develop. In addition, cotton regeneration via somatic embryogenesis is highly genotype-specific, and highly regenerable lines selected from the obsolete cultivar Coker 312 (Trolinder and Xhixian, 1989) serves as the industry standard at this time (Mishra *et al*., 2003).

To overcome the problems of regeneration and transformation of cotton somatic embryos, transformation of cotton using *Agrobacterium* and the shoot apical meristem has been studied (Zapata *et al*., 1999a; Satyavathi *et al*., 2002,

Satyavathi *et al*., 2005). The cotton cultivars transformed were three Indian varieties MCU5, DCH32, Coker 310FR (Satyavathi *et al*., 2002), a Texas cultivar CUBQHRPIS (Zapata *et al*., 1999a) and another Indian variety F846 (Satyavathi *et al*., 2005). Fertile transformed plants were produced and progeny analysis of these plants showed a classical Mendelian pattern of inheritance (Zapata *et al*., 1999a; Satyavathi *et al*., 2002; Satyavathi *et al*., 2005). Transformation frequencies were 1.47% for the Indian varieties (Satyavathi *et al*., 2002) and 0.8% for the Texas cultivar (Zapata *et al*., 1999a).

Since the shoot apical meristem can be regenerated into normal plants, this explant is ideal to use for transformation. Also, theoretically, the advantage of the shoot apex explant over other regeneration systems is that plants may be obtained from any genotype rather than from only those that regenerate from callus culture (Zapata *et al*., 1999b).

Using the shoot apical meristem regeneration system, transgenic progeny may be directly produced by transformation of cells in the LII layer (Marcotrigiano, 1990). The primary transformants will always be chimeric (Lowe *et al*., 1995; Zhong *et al*., 1996). The relatively stability of chimeric shoots depends on the original position of the transformed cell(s) and the competition between transformed and non-transformed cells (Marcotrigiano, 1990).

Successful transformation of shoot apical meristems by *Agrobacterium* has also been reported for other plant species including corn, (Gould *et al*., 1991b), banana

(May *et al*., 1995), rice (Park *et al*., 1996), sunflower (Alibert *et al*., 1999), lupin (Li *et al*., 2000) and pear (Matsuda *et al*., 2005). Normal fertile plants have been produced (Gould *et al*., 1991b; Park *et al*., 1996 Li *et al*., 2000). There have, however, been reports on the difficulty in recovering plants from *Agrobacterium*-transformed meristems (John and Stewart, 1992).

Transformation of elite cotton cultivars via particle bombardment of organized shoot tip meristems has been reported (Finer and McMullen, 1990; McCabe and Martinell, 1993; Chlan *et al*., 1995; Keller *et al*., 1997). Particle bombardment transformation protocols using the shoot apical meristem, although genotype-independent, are extremely laborious and the transformation frequencies for cotton by particle bombardment is low. Only 0.1 to 0.2% of bombarded meristems are recovered as transgenic plants (John, 1997) and the occurrence of chimeric plants within the regenerants is common (McCabe and Martinell, 1993). A large number of meristems have to be transformed and, if the cultivar is not amenable to induction of multiple shoots and axillary branching, the transformed status of the plants can be analyzed only in the next generation (John, 1997). Majeed *et al*. (2000) transformed shoot tip cultures using the combination of *Agrobacterium* and particle bombardment and reported 9.6% transformation efficiency.

Routine recovery of transgenic cotton plants using either *Agrobacterium* or particle gun has been restricted to a few genotypes (for example: Coker 312, CUBQHRPIS, Pima S-6, Sea Island) with no reports of transformed South

African commercially grown Sabie, LRCC 101, 107/1 or Palala cultivars. Therefore, development of an efficient and consistent regeneration and transformation protocol for these cultivars will greatly aid cotton transgenic technology development. Regeneration of Sabie, Palala, LRCC 101 and 107/1 plants from the shoot apical meristems has been developed (Chapter 5). These explants can therefore be used for transformation studies using *Agrobacterium* or particle bombardment.

The use of herbicides to control weeds allows growers to practice more efficient crop management, together with improving yields. Several classes of herbicides can be effective for broad-spectrum weed control, but these are either non-selective, killing also the crop plants, or they significantly injure some crops at the application rates required (Fox, 1990). Engineering herbicide tolerance into crops represents a new alternative for conferring selectivity of herbicides and enhancing crop safety. The production of herbicide resistant commercially-grown South African cotton plants to a single, highly efficient non-selective herbicide without toxicity to animals and rapid degradation in the soil would be welcomed, as these transgenic plants would provide more effective, less costly and more environmentally attractive weed control.

Two general approaches have been taken in engineering herbicide resistance or tolerance in plants. The first approach involves altering the level and sensitivity of the target enzyme for the herbicide and the second approach involves incorporating a gene that will detoxify the herbicide before it reaches the

biochemical target inside the plant cell (Stalker et al., 1988; Gasser and Fraley, 1992).

The herbicide resistance genes *bar* (Thompson *et al*., 1987) and *pat* (Bayer *et al*. 1972), have been widely used in transformation experiments (D' Halluin *et al*., 1992). The *pat* gene, isolated from *Streptomyces viridochromogenes* Tu494 (Bayer *et al*., 1972; Wohlleben *et al*., 1988; Alijah *et al*., 1991) encodes for the enzyme phosphinothricin acetyltransferase (PAT), which acetylates the free ammonium group of phosphinothricin (PPT), the active ingredient of the herbicide Basta, thereby rendering the latter non-toxic (Murakami *et al*., 1986; De Block *et al*., 1987; Thompson *et al*., 1987; Strauch *et al*., 1988). Transgenic PPT resistant plants expressing the *pat* or *bar* gene include soybean (Zeng *et al*., 2004), cotton (http://www.ogtr.gov.au), cassava (Sarria *et al*., 2000), oat and barley (Zhang *et al*., 1999b; Bregitzer and Tonks, 2003), sugar cane (Leibbrandt and Snyman, 2003), perennial grass (Shu *et al*., 2005) and carrots (Chen and Punja, 2002).

Genetically modified cotton has been approved for commercial use or trial in South Africa. These are all based either on *Bt* events or herbicide (glyphosate) tolerance. Over 90% of small-scale farmers grow GM cotton. *Bt* cotton has been planted by small-scale farmers in the Makhathini Flats on the northeast coast of South Africa (KwaZulu-Natal Province). The yields and profits from the GM cotton crops for small-scale farmers in South Africa have been boosted. To cotton farmers, growing *Bt* cotton would mean spending less money and time on

spraying crops with pesticides. These results could be significant for Africa's agricultural economy. Recent developments in gene transfer techniques provide an opportunity to modify commercially grown South African cotton cultivars by producing plants that are resistant to the herbicide Basta.

In view of the economic importance of cotton in South Africa and the potential to improve commercially grown cultivars by genetic transformation, a transformation protocol, using *Agrobacterium*-mediated DNA transfer and the shoot apical meristem was developed for South African cotton cultivars Sabie, 107/1, LRCC 101 and Palala.

6.2 Materials and methods

6.2.1 Explant preparation

Seeds of commercially grown South African cotton cultivars Sabie, Palala, LRCC 101 and 107/1 were obtained from the Tobacco and Cotton Research Institute, Rustenburg, South Africa. The seeds were washed briefly in soapy water and surface sterilized in 2.6% (v/v) sodium hypochlorite solution containing three drops of Triton X-100 per 200 ml for 20 min. Following this step and under sterile conditions, the seeds were placed in 70% (v/v) ethanol for 2 min. The seeds were then thoroughly rinsed four times in sterile distilled water. Following the rinsing steps, seeds were placed in sterilized petri dishes containing 20 ml of MS medium (Appendix A), pH 5.8. The seeds were germinated under growth

chamber conditions (25±2°C; 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}) for 5-7 days. After 5-7 days, shoot apical meristems measuring 0.5-1 mm in length were aseptically isolated from the *in vitro* grown cotton seedlings and used for plant regeneration on media containing antibiotics and PPT and for transformation trials.

6.2.2 Determination of the concentrations of antibacterial and selection agents for use in transformation experiments

Prior to transformation trials, the following were determined for cotton shoot apical meristems:

1) The concentrations of the antibacterial agents, namely carbenicillin and cefotaxime, which inhibited growth of *Agrobacterium*, but still allowed shoot regeneration from the shoot apical meristems. Sabie, Palala, LRCC 101 and 107/1 shoot apical meristems were placed onto cotton regeneration medium (Appendix F) containing 50 mg/l cefotaxime and 400 mg/l carbenicillin (Umbeck *et al*., 1987). Cotton explants were also placed onto the above medium containing no antibacterial agents for comparative purposes.
2) The minimum concentrations of the selection agents, namely kanamycin and PPT, which inhibited shoot regeneration and rooting. Shoot apical meristems were placed onto cotton regeneration medium (Appendix F) containing different concentrations (0, 25, 50, 100, 150 mg/l) of kanamycin and different concentrations (0, 0.1, 0.5, 1, 5, 20 mg/l) of PPT.

The cotton seeds were sterilized as described previously (6.2.1) and placed on MS medium for germination. After 5-7 days, seedlings were removed from the germination medium and placed into sterile petri dishes. Shoot apical meristems measuring 0.5-1 mm in length were aseptically isolated from the *in vitro* grown cotton seedlings. Ten shoot apical meristems from Sabie, Palala, LRCC 101 and 107/1 seedlings were placed on the media above in triplicate, the petri dishes sealed with parafilm and the explants incubated at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}. The explants were observed regularly for the formation of new shoots and the effect the various antibacterial agents and selective agents had on shoot formation. The explants were evaluated for colour and presence or absence of shoots. Regenerated shoots were then placed onto cotton rooting medium (Appendix F) containing the selective agents above and observed regularly for root development.

6.2.3 The effect on the production of shoots by injecting shoot apical meristems with sterile distilled water and acetosyringone

Shoot apical meristems were isolated from 5-7 day old seedlings (6.2.1), wounded three times with a needle and injected with 10 µl of sterile distilled water containing 20 µM acetosyringone. The needle used was the following size: 27 *g* x ½ inch. The wounded shoot apical meristems were then placed onto cotton regeneration medium (Appendix F) and the production of shoots was recorded over a period of three weeks. Twenty meristems for each cultivar were used.

6.2.4 Transformation of shoot apical meristems

Transconjugate *A. tumefaciens* containing pBI101 with the phosphinothricin acetyltransferase (*pat*) and the neomycin phosphotransferase (*npt II*) genes (Viljoen *et al*., 1995) was used for transformation experiments. This transformed *Agrobacterium* was maintained at 30°C on LB medium (Appendix B) containing 100 mg/l rifampicin (Sigma) and 100 mg/l kanamycin (Boehringer Mannheim). A colony from a freshly streaked plate was inoculated into 20 ml of YEP medium (Appendix B) containing 10 mM glucose (BDH), 20 µM acetosyringone (Aldrich) (Khan *et al*., 1994), 100 mg/l rifampicin and 100 mg/l kanamycin. The isolate was grown to the exponential phase (OD_{600nm} = 0.6-0.8) at 30°C with constant agitation at 160 rpm and then transferred to a centrifuge tube and centrifuged at 2 000 *g* for 15 min. The broth was decanted and the pellet resuspended in 18 ml LB broth (Appendix B) (OD_{600nm} = 0.6-0.8) with 20 µM acetosyringone and 10 mM glucose.

Two different transformation procedures were evaluated:

1. Five-seven day old shoot apical meristems measuring 0.5-1 mm in length were wounded three times with a sterile needle and inoculated with 10 µl of the 48 h transconjugate *Agrobacterium* culture. The explants were placed onto MS medium (Appendix A) for two days co-cultivation with the *Agrobacterium* at 25±2°C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}.

2. Five-seven day old shoot apical meristems measuring 0.5-1 mm in length were placed into the 48 h transconjugate *Agrobacterium* culture for 30 min and blotted dry on sterile filter paper. The explants were placed onto MS medium (Appendix A) for two days co-cultivation with the *Agrobacterium* at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 µE m^{-2} s^{-1}.

After two days co-cultivation with *Agrobacterium*, the transformed meristems were placed onto regeneration medium (Appendix F) containing 50 mg/l cefotaxime and 250 mg/l carbenicillin for one week and then transferred to regeneration medium (Appendix F) containing 50 mg/l cefotaxime and 250 mg/l carbenicillin, 1 mg/l PPT and 100 mg/l kanamycin for selection of transformed shoots. The explants were observed daily for shoot regeneration. For each transformation procedure, 100 meristems of Sabie, Palala, 107/1 and LRCC 101 were transformed.

Control (non-transformed, but wounded) shoot apical meristem explants were placed onto the following medium: medium without selection agents, medium with carbenicillin and cefotaxime and medium with carbenicillin, cefotaxime, PPT and kanamycin (concentrations of selection and antibacterial agents as above). Subcultures were made every two weeks to replenish depleted nutrients and antibiotics.

All putative transformed shoots (3-5 cm tall) from wounded and non-wounded meristem explants were excised from the shoot apical meristems and transferred to MS medium (Appendix A) containing 0.1 mg/l kinetin, 50 mg/l cefotaxime, 250 mg/l carbenicillin, 1 mg/l PPT and 100 mg/l kanamycin for elongation and selection of transformed shoots.

Transformation of meristems may result in chimeric plants. That is, only one cell in a cluster of meristematic cells might receive the foreign T-DNA, but the resulting enzymatic activity (transgene product) could protect the surrounding cells by kanamycin or PPT inactivation. As a result, a shoot could develop which would contain both transformed and non-transformed cells. As a first step in creating conditions to identify any possible non-transformed cells and cell-derivatives, a rooting regime in the presence of the selection agents, kanamycin and PPT, was used. Green, vigorously growing putative transgenic shoots were placed onto rooting medium (Appendix F) containing selection agents 100 mg/l kanamycin and 1 mg/l PPT, to induce rooting. Potential transgenic plants with healthy roots were transferred to sterile potting soil in tissue culture flasks and placed into the growth chamber for further growth at 25±2^{o}C with a 16:8 h light:dark photoperiod with light supplied at an intensity of 35-40 μE m^{-2} s^{-1}.

6.2.5 PCR analysis of transformed cotton shoots

To confirm integration of the *pat* gene in the genome of transformed cotton shoots after transformation, PCR analysis was performed. Total DNA from leaves of one

non-transformed cotton plant and new transformed shoots from all transformed plants was isolated using the cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). PCR was performed using *pat* gene specific primers which amplified the 558 bp *pat* coding region of the 1.3 kb chimeric gene construct. The PCR Core Kit (Boehringer Mannheim) was used for each PCR reaction. The PCR was carried out in a volume of 25 µl in a PCR tube.

Each reaction mixture contained 1X PCR reaction buffer [10 mM Tris-HCl, 50 mM KCL, pH 8.3 (20^oC)], 1.2 mM $MgCl_2$, 200 µM dNTP's (dATP, dCTP, dGTP, dTTP, pH 7.0), 0.5 µM of each of the 20 mer oligonucleotide primers (the sequences of the 20 mer oligonucleotide primers used were: left primer $^{5'}$ GTC TCC GGA GAG GAG ACC AG $^{3'}$ and right primer $^{5'}$ CCT AAC TGG CCT TGG AGG AG $^{3'}$) and 10 ng of template DNA. Sterile distilled water was added to give a total volume of 25 µl. The contents of the PCR tubes were mixed and centrifuged briefly at 13 000 *g*. The tubes were placed into the PCR machine and the template DNA denatured at 95^oC for 5 min. The tubes were placed on ice, centrifuged briefly at 13 000 *g* and then 0.5 units of *Taq* DNA polymerase added. After mixing the contents of the tube and a brief centrifugation at 13 000 *g*, 25 µl of sterile mineral oil was added to the tube. The tubes were placed back into the PCR machine and the DNA amplified by 30 repetitive cycles involving template denaturation (92^oC for 1 min), primer annealing (60^oC for 1 min) and extension (72^oC for 1 min). The DNA was then extended for 5 min at 72^oC. The reaction was stopped by placing the tubes on ice. Samples subjected to PCR included DNA from a transformed cotton plant, DNA from a non-transformed cotton plant

(negative control), water (negative control), pBI101/*pat* and 1.3 kb chimeric gene construct (positive controls). PCR products were separated on a 0.8% agarose gel stained with ethidium bromide to detect the amplified 558 bp fragment. To demonstrate the efficient separation of the DNA fragment, the DNA molecular weight marker VI was used.

6.3 Results

6.3.1 Determination of the concentrations of antibacterial and selection agents for use in transformation experiments

After 2-3 weeks on medium containing 50 mg/l cefotaxime and 400 mg/l carbenicillin, all cotton shoot meristems (100%) of the four cultivars tested produced shoots. All cotton explants placed onto medium without antibiotics also produced shoots. The explants were therefore not affected by the concentrations of the antibacterial agents included in the medium.

The percentages of shoot apical meristems of all four cotton cultivars producing shoots after 2-3 weeks on medium containing 0.1 mg/l PPT are presented in Table 6.1. At the higher concentrations of PPT (0.5, 1, 5, and 20 mg/l), the formation of shoots from all shoot apical meristems was inhibited when compared with the production of shoots from the meristem explants placed on medium without PPT. The explants became necrotic (Fig. 6.1). On medium without selection agents, new green shoots from 100% shoot apical meristems were observed. Therefore 1

Table 6.1: Percentage of shoot apical meristems of Sabie, 107/1, LRCC 101 and Palala producing multiple shoots on medium containing the selective agents PPT and kanamycin.

Cultivar	% of meristems producing shoots	
	0.1 mg/l PPT	25 mg/l kanamycin
Sabie	91.6	60
107/1	66.6	66.6
LRCC 101	72	59
Palala	53	69

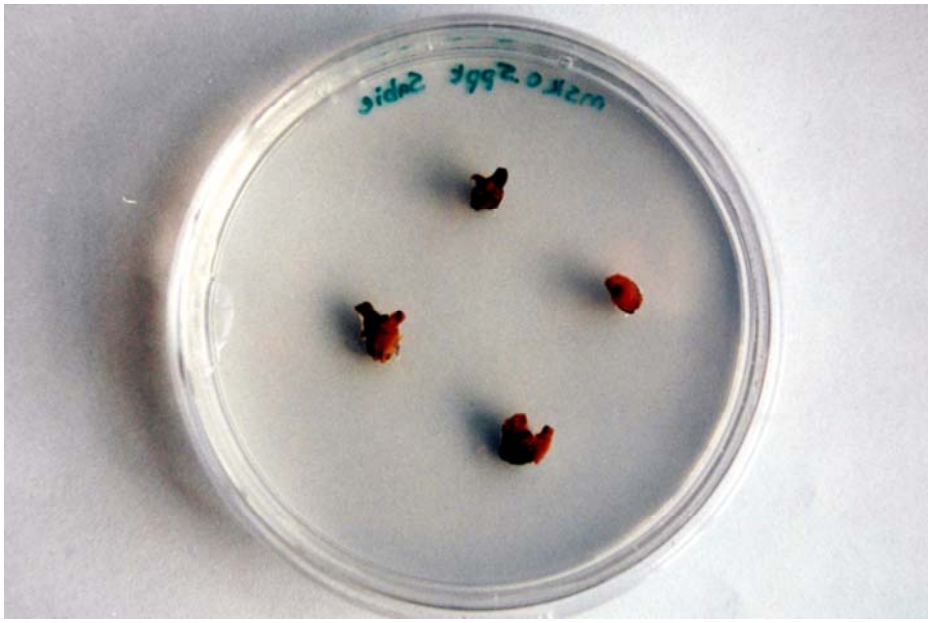

Figure 6.1: Necrotic shoot apical meristems of Sabie on cotton regeneration medium containing 0.5 mg/l PPT (*bar* 10 mm).

mg/l PPT was chosen for transformation experiments to select for transformed cotton explants.

The results of the percentages of shoot apical meristems of all four cotton cultivars producing shoots on medium containing 25 mg/l kanamycin are presented in Table 6.1. These shoots that had emerged from the shoot meristems of the cultivars began to show signs of chlorosis and the shoots eventually became necrotic by the third week. Cotton explants placed on medium containing 50, 100 and 150 mg/l kanamycin did not produce any shoots. Therefore, 100 mg/l kanamycin was chosen for transformation experiments to select for transformed cotton explants. Shoots placed onto rooting medium containing the above selection agents did not produce roots.

6.3.2 The effect on the production of shoots by injecting shoot apical meristems with sterile distilled water and acetosyringone

Prior to transformation experiments, the production of multiple shoots from the shoot apical meristems of all four cultivars, after wounding and injecting water and acetosyringone into the meristems, was assessed. All meristems produced green healthy shoots within two weeks on cotton regeneration medium (Fig. 6.2). After four weeks in culture, multiple shoots were produced.

Figure 6.2: Shoot induction from wounded shoot apical meristems of 107/1 (*bar* 10 mm).

6.3.3 Transformation of shoot apical meristems

During the initial seven days on non-selective medium, all co-cultivated and control explants retained a healthy green colour. After transfer to selection medium, 100% non-transformed shoot apical meristem explants turned brown and died within one week. There was no shoot production.

In contrast, transformed shoot meristems (wounded and non-wounded) of Sabie and 107/1, placed onto selection medium produced green, healthy shoots after two weeks (Fig. 6.3). One hundred percent shoot meristems of Palala and LRCC 101 did not produce transformed shoots and died within two weeks. Percentage of transformed shoot apical meristems producing multiple shoots was therefore 0% for Palala and LRCC 101.

The transformed shoot apical meristems of Sabie and 107/1 continued to undergo normal shoot development. This indicated that the shoot apices from which shoots were being produced in the presence of the selection agents, kanamycin and PPT, were transformed. The percentage of transformed wounded and non-wounded shoot apical meristems producing multiple shoots for Sabie and 107/1 are presented in Table 6.2.

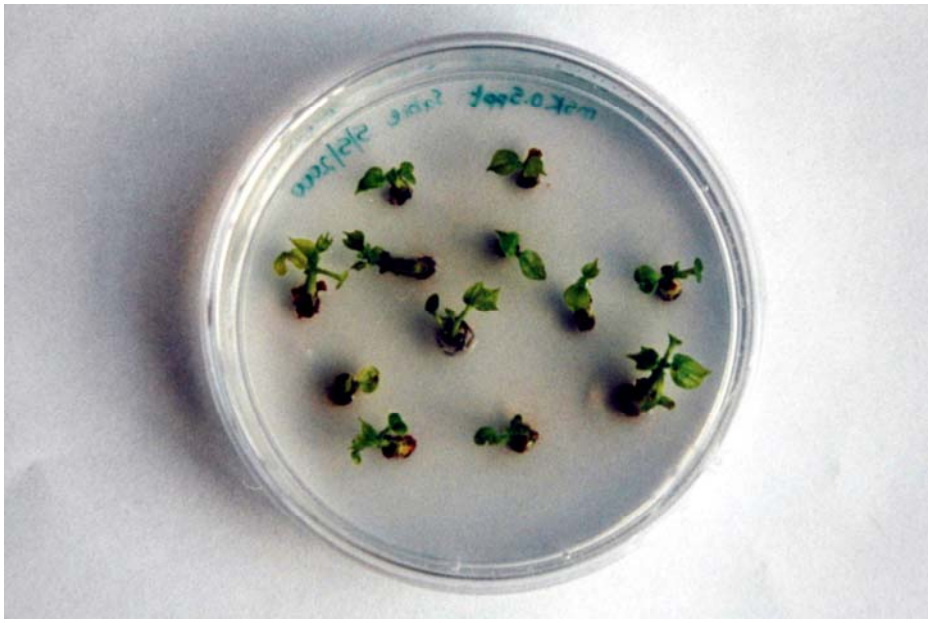

Figure 6.3: Shoot induction from transformed (wounded) Sabie shoot apical meristems on cotton regeneration medium containing 1 mg/l PPT and 100 mg/l kanamycin (*bar* 10 mm).

Table 6.2: Percentage of transformed wounded and non-wounded Sabie and 107/1 shoot apical meristems producing multiple shoots from the initial 100 transformed meristem explants.

Cultivar	% transformed apical meristems	No. shoots/meristem
Sabie (wounded)	34	5-6
Sabie (non-wounded)	15	4-5
107/1 (wounded)	25	4-5
107/1 (non-wounded)	12	3-5

All putative transformed shoots (3-5 cm tall) for each cultivar (from wounded and non-wounded shoot apical meristems) were placed onto elongation and rooting medium containing kanamycin and PPT. Results are presented in Table 6.3. Rooted shoots produced healthy transformed Sabie (Fig. 6.4) and 107/1 plants (Fig. 6.5). This indicated that the transformed Sabie and 107/1 plants contained the *pat* gene that was successfully detoxifying the herbicide PPT. The frequency of regenerated transformed plants from the initial 100 explants (wounded and non-wounded) to whole plants in tissue culture was therefore 3% and 2.3%, respectively, for Sabie, and 2.3% and 1.2%, respectively, for 107/1 (Table 6.3). Shoots that were not transformed did not produce roots and became necrotic within a week. Transformed Sabie and 107/1 plantlets were transferred to sterile potting mixture and acclimatized in the growth chamber. Once in the growth chamber all the transformed plantlets lost their leaves and died three weeks later.

Figure 6.4: Transformed Sabie shoot rooting on rooting medium containing 100 mg/l kanamycin and 1 mg/l PPT (*bar* 10 mm).

Figure 6.5: Transformed 107/1 shoot rooting on rooting medium containing 100 mg/l kanamycin and 1 mg/l PPT (*bar* 10 mm).

Table 6.3: Results of rooting of elongated transformed shoots from transformed shoot apical meristems of Sabie and 107/1.

	Wounded meristems		Non-wounded meristems	
	Sabie	107/1	Sabie	107/1
Initial no. shoots	187	113	68	48
No. rooted shoots	15	10	10	5
% rooted shoots	8	9	15	10
% transformed plants per meristem	3	2.3	2.3	1.2

6.3.4 PCR analysis of transformed cotton shoots

All transgenic Sabie and 107/1 plants were tested for the presence of the *pat* gene by PCR and the expected 558 bp fragment was present in the DNA of transformed leaves of all these plants, indicating successful transformation of meristematic tissues. The 558 bp fragment was present in the DNA of leaves of transformed Sabie (Fig. 6.6, lane 6) and 107/1 (Fig. 6.6 lane 7), positive controls, pBI101/*pat* and 1.3 kb chimeric gene construct (Fig. 6.6, lanes 3-4), but not in the DNA from the non-transformed cotton plant (Fig. 6.6, lane 5) and water sample (Fig. 6.6, lane 1). To rule out the possibility of chimeras, DNA from various leaves from the transformed Sabie and 107/1 plantlets were subjected to PCR. Figure 6.7 shows the results from DNA extracted from three leaves from one Sabie plantlet. DNA from all three leaves contained the *pat* gene as the expected 558 bp fragment was seen (Fig. 6.7, lanes 5-7). The expected 558 bp fragment was also present in the DNA of various leaves from the other transformed Sabie plants and 107/1 transformed plants.

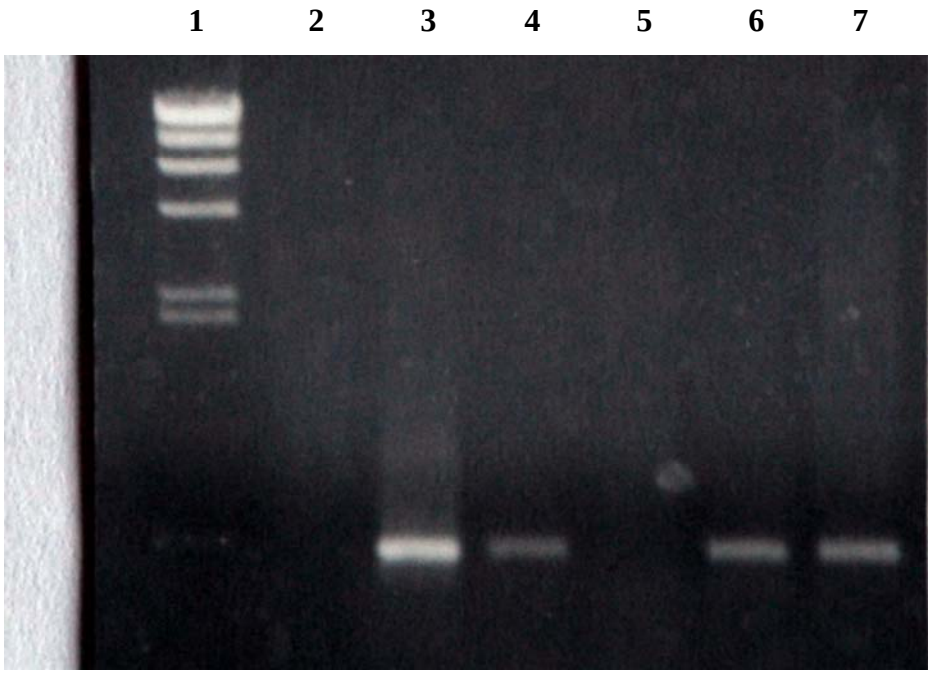

Figure 6.6: Amplification of the 558 bp *pat* coding region in transgenic Sabie and 107/1 leaves from one transformed Sabie and 107/1 plant. Samples of 25 µl were subjected to 30 cycles of amplification. Two microlitres of each sample were resolved on a 0.8% agarose gel and visualised by ethidium bromide fluorescence.

Lanes: (1) molecular weight marker VI; (2) negative control - water sample; (3) positive control - pBI101/*pat* DNA; (4) positive control - 1.3 kb chimeric gene construct; negative control – DNA from a non-transformed Sabie plant; (6) DNA from a transgenic Sabie plant; (7) DNA from a transgenic 107/1 plant.

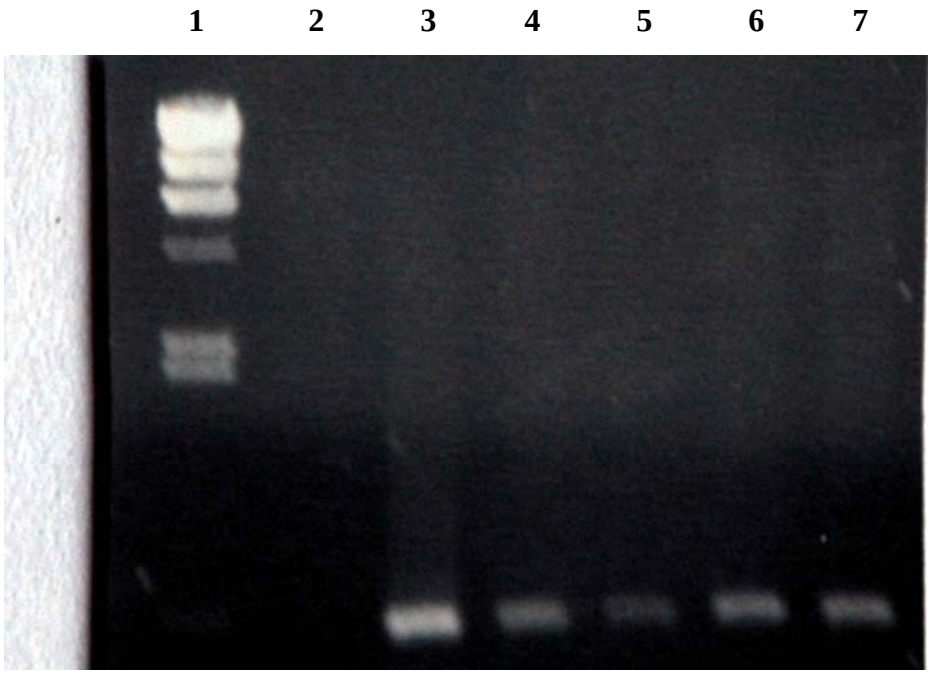

Figure 6.7: Amplification of the 558 bp *pat* coding region in three transgenic Sabie leaves from one transformed Sabie plant. Samples of 25 µl were subjected to 30 cycles of amplification. Two microlitres of each sample were resolved on a 0.8% agarose gel and visualised by ethidium bromide fluorescence.

Lanes: (1) molecular weight marker VI; (2) negative control – DNA from a non-transformed Sabie plant; (3) positive control - pBI101/*pat* DNA; (4) positive control - 1.3 kb chimeric gene construct; (5-7) DNA from a transgenic Sabie plant.

6.4 Discussion

6.4.1 Determination of the concentrations of antibacterial and selection agents for use in transformation experiments

When attempting transformation with *A. tumefaciens*, one of the most important steps is the selection of transformed cells from an abundance of non-transformed cells. Antibiotics are widely used in genetic transformation technology to select for transgenic tissues and/or to eliminate *Agrobacterium* from the cultures when its presence is no longer required (Estopa *et al*., 2001). Eliminating *A. tumefaciens* from cultures is important, because microbial contaminants in cultured plants can reduce multiplication and rooting rates or induce plant death (Cassells, 1991). Moreover, the elimination of *Agrobacterium* in transgenic plants is a pre-requisite in preventing the possibility of gene release when these plants are transferred to the soil (Barrett *et al*., 1997).

Carbenicillin, cefotaxime and ticarcillin, belonging to the B-lactam group, are the antibiotics most commonly employed to eliminate *Agrobacterium* from cultures after co-cultivation of plant tissues for transformation (Estopa *et al*., 2001). These antibiotics inhibit bacterial cell wall synthesis (Holford and Newbury, 1992). The effect of these antibiotics have been studied on different plant species (Estopa *et al*., 2001). The levels of antibacterial agents (50 mg/l cefotaxime and 400 mg/l carbenicillin) included in the regeneration medium for cotton in this study had no detrimental effect on the shoot regeneration response from shoot apical meristems.

Shoots were produced from 100% of Sabie, 107/1, LRCC 101 and Palala shoot apical meristems. Similarly, Satyavathi *et al*. (2002) reported that 400 mg/l cefotaxime had no effect on normal development of shoots from Indian cotton (cultivars MCU5, DCH32 and Coker 310FR) shoot apical meristems. Zapata *et al*. (1999a) reported that 250 mg/l clavamox did not have negative effects on shoot regeneration from the Texas cotton cultivar CUBQHRPIS, when shoot apical meristem explants were cultured.

Cefotaxime, a cephalosporin, provides broad spectrum anti-microbial activity without any significant toxicity to plant cells, is commonly used (Agrawal *et al*., 1998). Besides anti-microbial activity, cefotaxime has been reported to increase the frequency of shoot development and length of shoots from embryo-axes (Agrawal *et al*., 1998). Draper *et al*. (1988) reported that cefotaxime and carbenicillin had no effect on normal development of callus from various plant species. Besides antimicrobial activity, cefotaxime has been reported to influence morphogenesis in cotton cultures. It has growth promoting effects on embryo-axes (Agrawal *et al*., 1998). Agrawal *et al*. (1998) reported that cefotaxime influenced the frequency of shoot development, length of shoots and fresh weight of explants in all of the cotton cultivars tested, but the precise pattern of response varied between the cultivars. In contrast, in the present study, cefotaxime did not influence morphogenesis in any of the four cotton cultivars tested.

In order to evaluate the potential use of kanamycin and PPT as selection agents (double selection) for use in the selection of cotton explants, a determination of

the minimal concentrations of selection agents which inhibited regeneration of cotton was determined. In this study, at all concentrations of PPT higher than 0.1 mg/l, 100% cotton shoot apical meristems became necrotic. All shoots from cultivars Sabie, 107/1, Palala and LRCC 101 became necrotic and did not produce roots when 1 mg/l PPT was added to the rooting medium. From this result, 1 mg/l PPT was chosen for the selection of transformed herbicide resistant cotton shoot apical meristems in this study.

In this study, the level of sensitivity to kanamycin was 50 mg/l and higher for the cotton shoot apical meristems. From this result, 100 mg/l of kanamycin was chosen for selection of transformed shoots. Other researchers have reported on kanamycin levels of 100 mg/l (Zapata *et al*., 1999a) and 75 mg/l (Satyavathi *et al*., 2002) for the selection of transformed cotton shoot apical meristems. To be of use, the selective agent must exert stringent pressure on the plant tissue concerned. The effectiveness of a particular compound as a selective agent can also vary with the type of target explant or tissue to be selected, genotype and species (D'Halluin *et al*., 1992; Rathore *et al*., 1993; Estopa *et al*., 2001), with escapes (false-positive shoots) being one of the major problems. Double selection used in transformation protocols is more efficient in eliminating potential escapes (Chen and Punja, 2002). Kanamycin is one of the most widely used selection agents for plant transformation. The corresponding resistance gene (*npt II*) encodes an aminoglycoside 3'-phosphotransferase, which inactivates kanamycin by phosphorylation (Bowen, 1993).

6.4.2 Transformation of shoot apical meristems with *Agrobacterium*

In this study, the application of *Agrobacterium*-mediated genetic transformation to commercially grown South African cotton cultivars is described. The approach combined cotton's natural susceptibility to *Agrobacterium* infection with the ability of cotton meristematic cells to undergo regeneration. The choice of explant for *Agrobacterium*-mediated transformation is of extreme importance for successful transformation (D'Halluin *et al*., 1992). Shoot apical meristems were used as the starting material in this study because whole plants could be regenerated from the shoot apical meristems (Chapter 5). Using shoot apical meristems for transformation has several advantages over using immature embryos. First is the ability to obtain target tissues for transformation from dry seeds; the necessity to grow donor plants under controlled conditions is eliminated (Zhong *et al*., 1998). The second advantage is the direct vigorous regeneration of shoots from shoot apical meristems. Shoot regeneration is direct, relatively simple and needs less time to regenerate large numbers of plants (Saeed *et al*., 1997). Thirdly, plants regenerated from shoot apices are true to phenotype with low incidence of somaclonal variation and chromosomal abnormalities (Bajaj, 1998). The fourth advantage is that the shoot apex explant has few genotype limitations and is considered as more appropriate because meristematic cells are programmed for direct shoot organogenesis without an intervening callus or de-differentiation stage (Zhong *et al*., 1998; Zapata *et al*., 1999b). One of the disadvantages of using shoot apical meristems for transformation is that genotype of a seedling is unknown and commercially, the application of transformation is to

further improve superior (known) genotypes. Another disadvantage is that the primary transformants will always be chimeric (Lowe *et al*., 1995; Zhong *et al*., 1996). The relatively stability of chimeric shoots depends on the original position of the transformed cell(s) and the competition between transformed and non-transformed cells (Marcotrigiano, 1990).

In this study, effective selection for kanamycin and PPT resistance *in vitro* was shown, as well as the formation of shoots on the transformed shoot apical meristem explants of Sabie and 107/1.

Genetically engineered cotton plants have been obtained by *Agrobacterium*-mediated transformation of shoot apical meristems of selected cultivars (Zapata *et al*., 1999a; Satyavathi *et al*., 2002). The cultivars transformed were a Texas cultivar CUBQHRPIS (Zapata *et al*., 1999) and three Indian varieties MCU5, DCH32 and Coker 310FR (Satyavathi *et al*., 2002). This is the first report on the transformation of wounded shoot apical meristems of South African cotton cultivars, Sabie and 107/1, by *Agrobacterium*-mediated DNA transfer. In this study, a simple and rapid method for transforming cotton shoot apical meristems is described. One of the methods described in this study is technically different from previously reported methods using shoot apical meristems (Zapata *et al*., 1999a; Satyavathi *et al*., 2002). In these previously reported methods no wounding of the shoot apical meristems with a needle prior to incubation with *Agrobacterium* was performed. Zapata *et al*. (1999a) placed two drops of 5 µl of *Agrobacterium* (OD_{600}=0.8-0.9 nm) onto each shoot apex and then incubated the

shoot apices at 28°C under dark conditions for 48 h. Satyavathi *et al*. (2002) took shoot tip explants from 3-5 day old seedlings that were used directly or pre-cultured for two days on hormone free MS medium with B5 vitamins prior to infection and co-cultivation with the *Agrobacterium* culture (OD_{600}=0.6 nm). The explants were gently shaken in the bacterial suspension culture for about 20 min and then blotted dry on sterile filter paper, transferred to medium and co-cultivated under dark conditions for two days at 25°C.

Zapata *et al*. (1999a) reported 0.8% regenerated transgenic plants (cultivar CUBQHRPIS) from an initial 1010 *Agrobacterium*-treated shoot apices in a period of three months. Eight regenerated plants grew and were transferred to the soil, reaching maturity after approximately 4 months. The transgene (*GUS*) was observed in two successive generations from the regenerants. Satyavathi *et al*. (2002) reported that a total of 5094 shoot tip explants were co-cultivated in three cotton varieties (MCU5, DCH32 and Coker 310FR), out of which a total of 3418 (67%) survived on selection medium. Putative transgenic shoots rooted and about 75 transgenic plants were established. The percentage of regenerated transgenic plants recovered in 12-16 weeks from the time of gene transfer to establishment in pots was 1.47%. Higher frequencies (3% for Sabie and 2.3% for 107/1) of regenerated transgenic plants were obtained in 2-3 months in this study (from wounding the shoot meristem and then inoculating with *Agrobacterium*) when compared to the frequencies (0.8% and 1.47%) obtained in the study by Zapata *et al*. (1999a) and Satyavathi *et al*. (2002), indicating that the transformation protocol (involving wounding of the apical meristem) presented in this study is

possibly more efficient than previously reported methods and that transformation of Sabie and 107/1 was successful. However, the transgenic plants in the present study did not mature in potting mixture so frequency of regenerated transgenic plants established in soil could not be calculated. Future studies need to include protocols for improving acclimatization of transgenic cotton plants. As can be seen from the results obtained in this study, factors such as wound induction, contact time between the *Agrobacterium* and shoot apical meristem and selection procedures all affect transformation frequencies.

In this study, shoot apical meristems were inoculated with the *Agrobacterium* culture immediately after excision to allow efficient transformation of the plant cells. Transformed Sabie and 107/1 apical meristems produced shoots on medium containing 1 mg/l PPT, indicating that they were transformed and the *pat* gene was producing sufficient amounts of PAT to confer PPT-resistance under the selection conditions. Park *et al*. (1996) and Li *et al*. (2000) wounded the shoot apical meristems of rice and lupin, respectively, with a fine needle, to place bacteria in the shoot meristematic region. Park *et al*. (1996) reported that 2.8% of transformed shoot apices (overall 20 out of 721 shoot apices) survived on selection medium. Li *et al*. (2000) reported that approximately half of the putative transformation events produced transgenic seed. The overall transformation efficiency, as determined by the T_1 generation was between 0.05% and 0.75%. Their results indicated that extra wounding treatment with the hypodermic needle through the surface of the shoot apex may enhance stable transfer of T-DNA into the cells because access for *Agrobacterium* to a cell surface recognition site is

provided and the T-DNA can be integrated into the plant genome and transcribed into messenger RNA (Draper *et al*., 1988). Kudirka *et al*. (1986) reported that Peking explants inoculated 4 h after excision showed reduced frequency of transformation. Excision stimulates wound-associated cell divisions that make the plant cells competent for transformation as well as providing access for the bacterium to a cell surface recognition site. Plant cells must be undergoing genome replication and can be easily accessible to *Agrobacterium* to achieve transformation (Draper *et al*., 1988).

The percentage (34% for Sabie and 15% for 107/1) of transformed wounded cotton shoot apical meristems obtained in the present study was higher than the percentage (2.8%) of transformed wounded rice shoot apical meristems obtained in the study by Park *et al*. (1996). The percentage (3% for Sabie and 2.3% for 107/1) of regenerated transgenic plants per wounded meristem obtained in this study is higher than the overall percentage (0.05-0.75%) of transgenic lupin obtained by Li *et al*. (2000). Other researchers have obtained transgenic plants from *Agrobacterium*-mediated transformation of the shoot apical meristem. For example, D'Halluin *et al*. (1990) reported a low recovery of transformants from shoot meristems of alfalfa. Gould *et al*. (1981b) reported a transformation frequency of 0.075% from shoot meristems of corn. Matsuda *et al*. (2005) reported a transformation frequency of 4.8% for pear shoot meristem-explants. The differences in transformation frequencies obtained by *Agrobacterium*-mediated transfer differ between plant species and factors such as wound induction and selection protocols all affect transformation frequencies.

In order to enhance transformation rates by *Agrobacterium*-mediated DNA transfer, improvements have been made in the delivery of the bacterium (Bidney *et al*., 1992), and vectors have been modified to provide constitutive expression of *vir* genes (Ishida *et al*., 1996). Although transformation rates have been significantly improved using these modifications, increases in efficiency are still needed. The response of plants to wounding is an important factor in the interaction between *Agrobacterium* and plants (Potrykus, 1991). There have been reports were frequency of transformation mediated by *A. tumefaciens* can be greatly enhanced by first generating microwounds in the target tissue through high-velocity microprojectile bombardment with uncoated particles (Bidney *et al*., 1992; Knittel *et al*., 1994; Brasileiro *et al*., 1996) or particles coated with *Agrobacterium* (Cordero de Mesa *et al*., 2000), shaking with glass beads (Grayburn and Vick, 1995) or with carborundum (Cheng *et al*., 1996), sonication (Santarem *et al*., 1998) and scratching with sandpaper (Hoshi *et al*., 2004). Majeed *et al*. (2000) reported 9.6% transformation efficiency when cotton shoot tip cultures were transformed using a combination of *Agrobacterium* and particle bombardment. Further studies on enhancing transformation of commercially grown South African cotton cultivars could include some of the above-mentioned protocols and the addition of cysteine into the media during co-cultivation with *Agrobacterium* to increase the T-DNA delivery (Olhoft and Somers, 2001; Zeng *et al*., 2004).

In the present study, wounding of shoot apical meristems resulted in a higher percentage of transformed apical meristems of Sabie (34%) and 107/1 (25%)

when compared with the percentage (15% for Sabie and 12% for 107/1) obtained when shoot apical meristems were co-cultivated with *Agrobacterium*, but not wounded. However, these results are low compared to the results reported by Sayavathi *et al*. (2002) that 67% of non-wounded transformed shoot meristems survived on selection medium. A high concentration of kanamycin (100 mg/l) was used for selection in the present study, whereas Sayavathi *et al*. (2002) used 75 mg/l.

It has been reported that in the presence of a low concentration of acetosyringone, sugars such as glucose and galactose act synergistically as virulence-inducing agents during infection by *Agrobacterium* (Cangelosi *et al*., 1990; Fortin *et al*., 1992). Khan *et al*. (1994) also reported on the glucose and acetosyringone requirement in the co-cultivation medium for efficient gene transfer in clover. Similarly, Santarem *et al*. (1998) reported that the addition of 100 μM acetosyringone to the co-culture medium enhanced transient expression in transformed immature soybean cotyledons. Trick and Finer (1998) added 100 μM acetosyringone to the maintenance medium when transforming embryogenic suspension cultures with *Agrobacterium*. Olhoft and Somers (2001) added 200 μM acetosyringone to the co-culture medium when transforming soybean cotyledonary node cells. Based on these findings, the cotton explants in this study were co-cultivated in the presence of glucose and acetosyringone. The presence of these agents in the co-cultivation medium possibly had a positive effect on the transfer of the *pat* gene into the Sabie and 107/1 shoot apical meristem, hence the regeneration of the shoot apical meristem explants on medium containing

kanamycin and PPT. Future transformation studies without acetosyringone and glucose will need to be done to determine transformation efficiency with and without these agents.

The selectable marker used in previous shoot apical meristem transformation studies on cotton was *npt II* (Zapata *et al*., 1999a; Sayavathi *et al*., 2002). In this study, we used the *pat* and the *npt II* genes as selectable markers. To our knowledge, this is the first report on *Agrobacterium*-mediated transformation of shoot apical meristems of Sabie and 107/1 with the *pat* gene.

The 0% transformation frequency for LRCC 101 and Palala shoot apical meristems obtained in this study could have been attributed to inefficient transformation of regenerable cells, and/or poor selection or survival of such cells, or poor susceptibility to *Agrobacterium*. The recovery of transgenic plants may also involve cultivar regeneration potential on selective media (Meurer *et al*., 1998; Murray *et al*., 2004). It has been reported that during selection of transformants on medium containing selection agents, the majority of the cells in the explant die. Such dying cells may release toxic substances (such as phenolics) that in turn may impair regeneration of transformed cells. In addition, dying cells may form a barrier between the medium and the transgenic cells preventing or slowing uptake of essential nutrients (Joersbo and Okkels, 1996). The overall effect of necrotic tissue during selection is presumably reduced mitotic activity of the transformed cells resulting in less transgenic shoots emerging from the explants (Joersbo and Okkels, 1996). The 0% transformation frequency obtained

for LRCC 101 and Palala shoot apical meristems in this study possibly indicates that the transformation protocol described is genotype-dependent. In future studies more commercially grown South African cotton cultivars need to be evaluated to successfully determine whether this transformation protocol is genotype-dependent.

The presence of the *pat* gene in Sabie and 107/1 transformed plants was confirmed by PCR. No chimeric Sabie or 107/1 plants were obtained since leaves on all of these transformed plantlets contained the *pat* gene. Rooting in the presence of selection agents also eliminated the possibility of chimeric shoots that may have formed when the shoot apical meristems were transformed. May *et al*. (1995) reported that when shoot apical meristems of banana were transformed with *Agrobacterium*, 40% of the tissues which formed shoots on kanamycin demonstrated vigorous root growth when transferred to rooting medium containing kanamycin. They reported that the remaining 60% of the shoots were chimeric. Gould *et al*. (1981b) also reported on possible chimeras produced when shoot apical meristems of corn were injected with *Agrobacterium*.

In this study, for example, only 15 transformed shoots out of 187 obtained from wounded Sabie meristems, rooted in the presence of kanamycin and PPT and formed plantlets. This data possibly indicates that the remaining shoots were chimeric, and did not contain meristematic tissues that would give rise to PPT-resistant roots.

Due to the fact that transformed cotton plants died and were not acclimatized in the growth chamber, no seed was produced and no more leaves were available for extraction of plant DNA. As a result, no Southern blotting or herbicide application experiments could be performed. Further molecular analysis is needed to determine integration, expression and stability of the *pat* gene in transformed shoot apical meristems and in the progeny. Herbicide application studies are needed to establish the percentage of herbicide tolerant plants.

In this study, transgenic herbicide resistant Sabie and 107/1 plantlets were obtained in 2-3 months. Once the transformed plantlets were transferred to sterile potting mixture in tissue culture flasks, all the transformed plantlets lost their leaves and died. When non-transformed plants were subjected to exactly the same method, they continued to grow into mature plants. The reason that mature transgenic cotton plants were not produced was not due to the tissue culture protocol, but possibly the result of transformation. McKenzie and Cress (1992) reported that no mature transgenic soybean plants were obtained when cotyledons were transformed with *Agrobacterium* because of problems acclimatizing the transgenic shoots. The plantlets failed to grow unless their stems had elongated sufficiently before rooting. Future work needs to be done to increase the recovery of mature transgenic plants in the soil.

Transformation of elite cotton cultivars via particle bombardment of organized shoot tip meristems has been reported (Finer and McMullen, 1990; McCabe and Martinell, 1993; Chlan *et al*., 1995; Keller *et al*., 1997). Particle bombardment

transformation protocols using the shoot apical meristem, although genotype-independent, are extremely laborious and the transformation frequencies for cotton by particle bombardment is low. Only 0.1 to 0.2% of bombarded meristems are recovered as transgenic plants (John, 1997) and the occurrence of chimeric plants within the regenerates is common (McCabe and Martinell, 1996). When compared to the higher transformation frequencies of 1.2-3% obtained in this study and 0.8% and 1.47% obtained in other studies (Zapata *et al*., 1999a; Satyavathi *et al*., 2002), it seems evident that *Agrobacterium*-mediated transformation of cotton shoot apical meristems is possibly more efficient than particle bombardment.

The results represented here demonstrate that shoot apical meristem tissues from Sabie and 107/1 cotton seedlings can be used for *Agrobacterium*-mediated DNA transfer. However, it should be noted that no transformation system is applicable to all genotypes, as demonstrated in this study. Transformed Sabie and 107/1 shoot apical meristems were obtained, but no transformed Palala and LRCC 101 shoot apical meristems. This method may possibly be extended to other cotton cultivars and can be applied to transform cotton with genes that have economical importance.

6.5 Conclusions

In conclusion, the commercially grown South African cotton cultivars, Sabie and 107/1, have been genetically transformed with the *pat* gene by a rapid, simple and

possibly genotype dependent *Agrobacterium*-mediated transformation protocol. The overall frequency of regenerated transgenic Sabie plants per meristem was 3% and 2.3% for wounded and non-wounded meristems, respectively. The overall frequency of regenerated transgenic 107/1 plants per meristem was 2.3% and 1.2% for wounded and non-wounded meristems, respectively. The results obtained in this study are promising since data on transformation of South African cultivars of cotton are not available. The presence of weeds in cotton fields is one of the main causes of productivity loss and the generation of herbicide-resistant cotton varieties is a significant step towards the genetic improvement of cotton. This is the first report of *Agrobacterium*-mediated cotton transformation using the *pat* gene and shoot apical meristems of the cultivars Sabie and 107/1. The transformation methodology developed in this study will thus be useful for future cotton crop improvement programmes in South Africa.

CHAPTER 7

CONCLUSIONS

The conclusions from the studies on tobacco (*Nicotiana tabacum*), soybean (*Glycine max*) and cotton (*Gossypium hirsutum*) are presented in this chapter.

In this study, *Agrobacterium*-mediated DNA transfer, and the efficiency of regeneration of tobacco plants from leaf disks, facilitated the production of nine herbicide resistant tobacco plants. Transformed tobacco plants were obtained following *Agrobacterium*-mediated DNA transfer of the *pat* gene in the presence of 20 μM acetosyringone. PCR analysis confirmed the presence of the *pat* gene in the nine transformed tobacco plants. The transformed plants did not differ phenotypically from the non-transformed plants, indicating that the existence and expression of the *pat* gene did not have any deleterious effects on growth characteristics. The transformed plants grew to maturity, flowered and produced seed. The *pat* gene was present in the T1, T2 and T3 progenies of the transformed tobacco plants indicating stable inheritance, integration and expression of the *pat* gene. Transgenic tobacco plants and T1 transformed progeny sprayed with a 2% aqueous solution of the commercial herbicide Basta, remained green and were fully tolerant to the herbicide. Cycles of propagation, temperatures of 30^{o}C, 35^{o}C and 40^{o}C and drought stress did not have an effect on the stable expression of the *pat* gene in the transgenic tobacco plants. The stable expression of transgenic phenotypes is essential for the successful commercialisation of transgenic crops and this study was successful in demonstrating the stability of a transgene through three generations, six cycles of propagation and environmental stress.

It is also necessary to combine molecular analysis and field evaluation of individual transgenic lines. Field trials involving the herbicide tolerant tobacco plants will, therefore, need to be done to evaluate yield and expression of herbicide resistance under varying environmental conditions.

In view of the economic importance of South African commercially grown soybean and cotton cultivars, and the potential to improve these cultivars by genetic transformation, the development of a reliable regeneration method is of paramount importance. In this study, a reasonably successful regeneration protocol was developed for the production of plants from isolated shoot apical meristems of soybean and cotton seedlings.

The results of the logistic regression showed that the type of cytokinin (BA, TDZ, kinetin or zeatin) tested and the concentration of that cytokinin had a significant effect on the production of shoots from the shoot apical meristems of Talana and Ibis. The type of cultivar did not have a significant effect on the production of shoots from the shoot apical meristems at different concentrations of the cytokinin and also at the different cytokinins tested.

The highest percentage of Talana (62%) and Ibis (57%) shoot apical meristems producing multiple shoots (6-7 per meristem) was on regeneration medium containing 0.05 mg/l BA. The highest percentage of Talana (46%) and Ibis (39%) shoot apical meristems producing multiple shoots (6-7 per meristem for Talana and 5-6 for Ibis) was on regeneration medium containing 1 mg/l TDZ. Therefore,

the best cytokinin and concentration for shoot organogenesis from shoot apical meristems of Talana and Ibis is 0.05 mg/l BA. As both cultivars responded uniformly on medium containing varying concentrations of BA and TDZ, this regeneration system appeared to be genotype independent. Kinetin and zeatin did not induce shoots from the shoot apical meristems and are therefore not effective cytokinins for soybean organogenesis from the shoot apical meristem of Talana and Ibis. All regenerated shoots from shoot apical meristems cultured on BA or TDZ alone did not form roots on rooting media used in this study and no regenerated plantlets were produced. Future work could include evaluating other rooting media. The effect of combinations of these cytokinins used in this study on the regeneration of shoots from the shoot apical meristems could also be assessed, as well as combinations with various auxins to improve rooting and plant regeneration frequencies.

The establishment of a rapid and reproducible plant regeneration system from the shoot apical meristems of two commercially grown soybean cultivars, Talana and Ibis, was successfully achieved. This is the first report of a successful regeneration protocol for the South African soybean cultivars Talana and Ibis. The procedure involves the use of both a cytokinin (BA) and auxin (NAA) in the regeneration medium. Plant regeneration frequencies per meristem of 66% and 52% were obtained for Talana and Ibis, respectively. All regenerated plants were phenotypically normal, fertile and set seed. The plant regeneration protocol appeared to be genotype independent. The success of the current work on the regeneration of South African soybean cultivars from shoot apical meristems and

the reasonably high frequency of plant regeneration indicates that soybean plant tissue culture can provide the opportunity for genetic engineering and crop improvement of soybean. Other commercially grown South African soybean cultivars need to be evaluated for their regeneration potential in future studies.

The results of the logistic regression showed that the type of cytokinin (BA, TDZ, kinetin or zeatin) tested and the concentration of that cytokinin had a significant effect on the production of shoots from the shoot apical meristems of Sabie, 107/1, LRCC 101 and Palala. The type of cultivar also had a significant effect on the production of shoots from the shoot apical meristems at different concentrations of the cytokinin and also at the different cytokinins tested.

The highest percentage (90-100%) of shoot apical meristems of Sabie, 107/1, LRCC 101 and Palala producing multiple shoots was on medium containing either kinetin or zeatin. It was therefore concluded that they are the best cytokinins for shoot organogenesis from the shoot apical meristems of all four cotton cultivars. The concentrations of kinetin which produced the highest percentage (100%, 93.3%, 99.3% and 100% respectively) of shoot apical meristems of Sabie, LRC 101, 107/1 and Palala producing multiple shoots, were 0.1 mg/l, 0.05 and 1.0 mg/l, 1 mg/l and 0.5 mg/l, respectively. The number of shoots produced per meristem at these concentrations was 5-6 for Sabie and Palala, 3-4 for LRCC 101, 4-5 for 107/1. The concentrations of zeatin which produced the highest percentage (95.3%, 99.3%, 99.3% and 94% respectively) of shoot apical meristems of Sabie, LRCC 101, 107/1 and Palala producing multiple shoots, were

0.01 mg/l, 0.05 mg/l, 0.01 mg/l and 0.05 mg/l, respectively. The number of shoots produced per meristem at these concentrations was 3-5 for Sabie, LRCC 101 and 107/1 and 3-4 for Palala. The highest percentage (85.3%, 82%, 66% and 80.6%, respectively) of shoot apical meristems of Sabie, LRCC 101, 107/1 and Palala producing multiple shoots was on medium containing BA at concentrations of 0.05 mg/l for Sabie and LRCC 101, 0.10 mg/l for 107/1 and 0.01 mg/l for Palala. The number of shoots produced per meristem at these concentrations was 3-4 for Palala and LRCC 101 and 3-5 for Sabie and 107/1. The highest percentage of shoot apical meristems producing multiple shoots was at different concentrations of TDZ for the different cultivars: 0.01 mg/l for Sabie (80.6%), 0.01 mg/l for LRCC 101 (46%), 0.05 mg/l for 107/1 (55.3%) and 0.05 mg/l for Palala (66%). Media containing 0.5 mg/l or more TDZ caused loss of apex organization and promoted callusing of the shoot meristem for cultivars Sabie, 107/l and Palala. The shoots that were produced from the explants (2-3 per meristem for Palala and 107/1 and 2 per meristem for Sabie and LRCC 101) after four weeks at the lower concentrations of TDZ were small (>0.5 cm), did not elongate and eventually showed signs of chlorosis and became necrotic. TDZ was therefore concluded to be an ineffective cytokinin for shoot production from shoot apical meristems of the four cotton cultivars. Shoot regeneration from the shoot apical meristems appeared to be genotype dependent at the different concentrations of cytokinins. Root initiation from shoots of all four cotton cultivars did not occur on half-strength MS medium with or without 0.05 mg/l and 0.1 mg/l NAA or IBA. These hormone treatments resulted in tissue mortality. Rooting of elongated Sabie, Palala, LRCC 101 and 107/1 shoots (3-5 cm)

occurred on full strength MS medium containing 0.1 mg/l kinetin. The overall frequency of regenerated cotton plants per meristem was 90% for Sabie, 74.6% for LRCC 101, 69.5% for 107/1 and 70% for Palala. Phenotypically normal regenerated cotton plants bore flowers and bolls and produced viable seed. The effect of combinations of the cytokinins used in this study on the regeneration of shoots from the shoot apical meristems could also be assessed in the future, as well as combinations with various auxins to improve plant regeneration frequencies.

The establishment of a rapid, efficient and simple plant regeneration system from the shoot apical meristems of the commercially grown cotton cultivars, Sabie, 107/1, Palala and LRCC 101, was successfully achieved. The results obtained in this study are promising, since data on the regeneration of the commercially grown South African cotton cultivars Sabie, LRCC 101, Palala and 107/1 are not available. This is the first report of a successful regeneration protocol producing a high frequency of regenerated commercially grown South African cotton plants. Future work could include evaluating other cotton cultivars for their regeneration potential. This shoot apical meristem-based regeneration method can be applied to plant transformation, either by particle bombardment or *Agrobacterium*-mediated DNA transfer for the introduction of foreign genes into commercially grown South African cotton cultivars for crop improvement.

Three parameters in this study were critical in developing the soybean transformation protocol: (1) the use of cultivars susceptible to *Agrobacterium*

transformation, (2) the development of a regeneration response from soybean explants and (3) sufficient sensitivity of cells to kanamycin and PPT. The commercially grown South African soybean cultivars, Talana and Ibis, showed susceptibility to the virulent *A. tumefaciens* strain sis 43. Tumours that formed on these plants were cultured *in vitro*. It was therefore established that Talana and Ibis are compatible hosts for *A. tumefaciens*.

This study provided the first report on the regeneration of transgenic shoots and recovery of transgenic plants in tissue culture from transformed shoot apical meristems of Talana. No transformed Ibis shoot apical meristems were obtained. Transformation of Talana shoot apical meristems with the *pat* gene occurred via *Agrobacterium*-mediated DNA transfer in the presence of 20 μM acetosyringone and 10 mM glucose. Shoot apical meristems were first wounded with a needle and then incubated with the *Agrobacterium*. The incorporation of 100 mg/l kanamycin and 1 mg/l PPT in the selection medium inhibited the regeneration of non-transformed shoot apical meristems, but permitted shoot regeneration from the transformed shoot apical meristems, indicating that the *pat* gene was present and successfully detoxifying the herbicide PPT. The presence of the *pat* gene in many shoots from the transformed Talana plants was confirmed by PCR, indicating successful transformation of meristematic tissues and the presence of non-chimeric plants. The overall transformation frequency of transformed Talana plants per shoot apical meristem was 1.06%. The yield was eight transgenic Talana plants that did not acclimatize in the potting mixture and eventually died.

This is the first report of herbicide (Basta) resistant Talana plants obtained via *Agrobacterium*-mediated DNA transfer of the shoot apical meristem.

Commercially grown South African cotton cultivars, Sabie and 107/1, have been genetically transformed with the *pat* gene by a rapid, simple and possibly genotype dependent *Agrobacterium*-mediated transformation protocol. The successful transformation of the Sabie and 107/1 shoot apical meristems with the *pat* gene occurred via *Agrobacterium*-mediated DNA transfer in the presence of 20 μM acetosyringone. Shoot apical meristems were wounded with a needle and then inoculated with the *Agrobacterium*. The overall transformation frequencies of transformed plants in tissue culture per meristem were 3% for Sabie and 2.3% for 107/1. Non-wounded shoot apical meristems incubated with *Agrobacterium* produced overall transformation frequencies of transformed plants per meristem of 2.3% for Sabie and 1.2% for 107/1. Wounding of the shoot apical meristems prior to incubation with the *Agrobacterium* therefore produced higher transformation frequencies than non-wounding of explants. The incorporation of 100 mg/l kanamycin and 1 mg/l PPT in the selection medium inhibited the regeneration of non-transformed shoot apical meristems, but permitted shoot regeneration from the transformed shoot apical meristems, indicating that the *pat* gene was present and successfully detoxifying the herbicide PPT. The presence of the *pat* gene in the DNA of many shoots from transformed Sabie and 107/1 plants was confirmed by PCR, indicating successful transformation of meristematic tissues and the presence of non-chimeric plants. However, the transgenic plants did not acclimatize in the potting mixture and eventually lost their leaves and

died. This is the first report of herbicide (Basta) resistant Sabie and 107/1 plants obtained via *Agrobacterium*-mediated DNA transfer of the shoot apical meristems. The results obtained in this study are promising, since data on transformation of South African cultivars of cotton are not available.

The presence of weeds in soybean and cotton fields is one of the main causes of crop productivity loss. Herbicide (Basta) resistant Talana, Sabie and 107/1 plants were achieved with some success. The potential exists for herbicide resistant soybean and cotton crops to take agriculture in a new direction, by providing the solution to effective weed control. The availability of these crops could improve weed control by providing flexibility in the timing of herbicide applications and by reducing overall herbicide use. The transformation methodology developed in this study will thus be useful for future soybean and cotton crop improvement programmes in South Africa. This method can be extended to other soybean and cotton cultivars and can be applied to transform soybean and cotton with genes that have economical importance.

Future research on the transformation of shoot apical meristems of the soybean and cotton cultivars used in this study and other soybean and cotton cultivars, could include the addition of cysteine into the media during co-cultivation *with Agrobacterium* to increase the T-DNA delivery, improving selection and regeneration protocols and increasing the wounding of explants by microprojectile bombardment or sonication, to increase the frequency of fertile transgenic soybean and cotton plants by *Agrobacterium tumefaciens*.

In this study, the most critical factor in transformation of soybean and cotton is the acclimatization of transgenic plantlets in the soil. Acclimatization is a huge stumbling block that can reduce success rates of transformation. Future work needs to be done to increase the recovery of mature transgenic soybean and cotton plants in the soil. Acclimatizing the transgenic plants by placing transgenic plantlets into sterile potting mixture in pots (instead of tissue culture flasks) and enclosing with glass jars could also be assessed.

To fully evaluate and characterize the stability of the transferred *pat* gene in the transformed cotton and soybean plants, foreign gene expression will have to be examined in the progeny. The *npt II* and *pat* genes were efficient selectable markers. The effective selectivity was probably due to the efficient translocation of PPT and/or the tendency of PPT to accumulate in fast growing tissue. An effective selection system makes transformation experiments more efficient and cost effective. Future work could involve linking the *pat* gene with genes that protect plants against viruses, pathogens and insects. The presence of the latter gene could be easily monitored by the expression of the *pat* gene. A marker gene like *pat* would therefore be a valuable tool for the breeder to use alongside other genes in his breeding programmes.

In summary, soybean and cotton plants were successfully regenerated *in vitro* from shoot apical meristems at appreciable high frequencies and therefore, transfer of appropriate trait genes into these explants can be applied by *Agrobacteriu*m- inoculation or particle gun acceleration. The success of this

study on the regeneration and transformation of soybean and cotton indicates that South African agriculture now has available techniques for plant regeneration and recombinant DNA technology for crop improvement of soybean and cotton.

Genetic engineering has great potential for accelerating crop improvement and has already yielded encouraging results. The successful use of transgenic approaches to combat pests and diseases and malnutrition among the poor masses constitutes a major breakthrough in the genetic amelioration of crop plants (Jauhar, 2001). However, as with any new technology, genetic engineering is encountering resistance from certain sections of the public. Campaigns are being waged to create concerns and even fear about the potential adverse impact of genetically modified organisms or foods on human health and the environment. A perceived, or perhaps even real concern, is the potential for a transgene to move from a crop plant to its wild relative, thereby creating the possibility of producing a "super weed" that may be hard to control by the use of available herbicides (Jauhar, 2001). In most cases this is unlikely to happen because of the difficulty of hybridization between a transgenic crop plant and its weedy relative.

Another concern is the possibility of health risks posed by GM foods, e.g. allergenicity. The genetically modified foods, must, therefore, be carefully tested before release to the public. Although the issues, concerns and possible misconceptions of certain groups may be overblown, some concerns may be genuine because no technology is completely risk-free and, therefore, we must

weigh potential benefits against any possible adverse effects of the new technology for crop improvement.

In South Africa, in 2005, the Genetically Modified Organisms (GMOs) Act was to be amended to improve certain aspects and to ensure compliance with the Biosafety Protocol (http://www.africabio.com). Lobbyists from industry, research institutions and environmental organizations urged legislators to reconsider the amendments suggested by the agricultural department. Suggestions were highly polarized, with environmental groups calling for tighter control and the industry and research groups calling for legislation that would not stifle South Africa's fledging biotechnology industry. Biotechnology is very important for Africa, where up to three-quarters of the workplace is involved in agriculture, and 70% of the population depends on farming as their only source of income (http://www.agricabio.com). Estimates showed the demand for maize alone was set to rise 80% by 2020 in sub-Saharan Africa (http://www.agricabio.com). Agricultural biotechnology offers the prospect of more insect, virus and fungal-resistant maize crops, as well as the opportunity to make them drought tolerant. Regulations and legislation therefore need to provide safety checks and balances, but remain easy to use by all, including scientists and farmers. The cost of biosafety assessments also need to be minimized to ensure maximum benefits from the technology (http://www.agricabio.com).

I believe that genetically modified crops in conjunction with conventional practices offer a safe and effective technology that can contribute to a better

environment and more sustainable and productive agriculture. Plant biotechnology, where herbicide resistance is established in various crop plants, will have economic, health and environmental gains. Transgenic soybean and cotton grown in South Africa will benefit small-scale and commercial farmers economically. Soybean is an important crop in South Africa. In addition to animal feed requirements, demands for a cheap source of protein by our expanding population have resulted in a sharp increase in South African soybean production over the past few years. In South Africa, over 90% of small-scale farmers grow genetically modified cotton. These are all based either on *Bt* events or herbicide (glyphosate) tolerance. *Bt* cotton has been planted by small-scale farmers in the Makhathini Flats on the northeast coast of South Africa. To cotton farmers, growing *Bt* cotton would mean spending less money and time on spraying crops with pesticides. These results could be significant for Africa's agricultural economy. Biotechnology needs to be explored but at the same time to protect against possible risks of biotechnology. I believe that with more education and awareness, this technology will find acceptance throughout the world. Hopefully, the huge potential of genetic engineering will be harnessed to the best advantage of the entire human race.

CHAPTER 8

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A P P E N D I C E S

A P P E N D I X A

Murashige and Skoog (1962) medium (MS)

4.3 g/l MS basal salt mixture (Sigma)
1 ml/l of 1000X MS vitamin stock solution (Sigma)
30 g/l sucrose (BDH)
8 g/l purified agar (Oxoid)
pH 5.7

For ½ MS medium, the MS basal salts were used at half of the above concentration with full vitamins.

Gamborg's B5 (B5) medium

3.1 g/l B5 basal salts (Sigma)
1 ml/l of 1000X Gamborg's vitamin stock solution (Sigma)
30 g/l sucrose (BDH)
6 g/l purified agar (Oxoid)
pH 5.7

Schenk and Hildebrandt (SH) medium

3.2 g/l SH basal salts (Sigma)
1 ml/l of 1000X MS vitamin stock solution (Sigma)
30 g/l sucrose (BDH)
6 g/l purified agar (Oxoid)
pH 5.7

For seed germination on ½ SH medium, the basal salts were used at half of the above concentration.

A P P E N D I X B

Luria-Bertani (LB) broth and LB agar medium

10 g/l bacto-tryptone (Biolab)
5 g/l bacto-yeast extract (Biolab)
10 g/l NaCl (Associated Chemical Enterprises cc)
pH 7.0

For medium containing agarose, 15 g/l agar commercial gel (Biolab) was added before autoclaving.

YEP medium

16 g/l bacto-peptone (Biolab)
16 g/l NaCl (Associated Chemical Enterprises cc)
8 g/l yeast extract powder (Biolab)
pH 7.5

YMCR medium

10 g/l mannitol (Saarchem)
0.5 g/l K_2HPO_4 (Saarchem)
0.2 g/l $MgSO_4.7H_2O$ (Saarchem)
0.1 g/l NaCl (Associated Chemical Enterprises cc)
0.4 g/l yeast extract (Biolab)
15 g/l agar commercial gel (Biolab)
1 ml/l congo red dye (from stock of 0.2 g/100ml)
pH 6.8-7.0

A P P E N D I X C

Components of potting mixture

35 l sterile soil
15 l peatmoss
10 l coarse sand
50 g chalk
120 g base (10 l vermiculite, 10 l superphosphate, 5 l sulphate of potash).

Components of Multifeed (Plaaskem)

Multifeed Classic is a concentrated, completely water soluble fertilizer which is readily taken up by the plant and which is ideally suited for optimum root development of seedlings.

Nitrogen 190 g/kg
Phosphorus 82 g/kg
Potassium 158 g/kg
Magnesium 900 mg/kg
Zinc 350 mg/kg
Boron 1000 mg/kg
Molybdenum 70 mg/kg
Iron 750 mg/kg
Manganese 900 mg/kg
Copper 75 mg/kg

Weekly application: dissolve 1 measuring spoon (5 g) in 2 l water and apply.
Drench soil with water after application.

A P P E N D I X D

20X SSC

175.3 g/l NaCl (Associated Chemical Enterprises cc)
88 g/l Tri-sodium citrate (BDH)
pH 7.0

A P P E N D I X E

Hormone-free PC-L2 medium

21 mg/l potassium nitrate
1 mg/l potassium iodide
5 mg/l zinc sulphate heptahydrate
0.1 mg/l cobalt chloride hexahydrate
0.1 mg/l cupric sulfate pentahydrate
15 mg/l manganese sulfate monohydrate
435 mg/l magnesium sulfate heptahydrate
600 mg/l calcium chloride
1 g/l ammonium nitrate
5 mg/l boric acid
25 mg/l ferrous sulfate EDTA heptahydrate
85 mg/l sodium phosphate monobasic anhydrous
325 mg/l potassium phosphate
0.4 mg/l sodium molybdate dihydrate
1 mg/l thiamine hydrochloride
0.5 mg/l pyridoxine hydrochloride
250 mg/l myo-inositol
25 g/l sucrose (BDH)
8 g/l purified agar (Oxoid)
pH 5.8

Soybean shoot regeneration medium

4.3 g/l MS basal salt mixture (Sigma)

1 ml/l of 1000X MS vitamin stock solution (Sigma)

20 g/l sucrose (BDH)

7 g/l purified agar (Oxoid)

0.05 mg/l BA (Sigma)

0.2 mg/l NAA (Sigma)

pH 5.8

A P P E N D I X F

Cotton shoot regeneration medium

4.3 g/l MS basal salt mixture (Sigma)
1 ml/l of 1000X MS vitamin stock solution (Sigma)
20 g/l sucrose (BDH)
7 g/l purified agar (Oxoid)
0.01 mg/l kinetin (Sigma)
pH 5.8.

Rooting medium for cotton shoots

4.3 g/l MS basal salt mixture (Sigma)
1 ml/l of 1000X MS vitamin stock solution (Sigma)
20 g/l sucrose (BDH)
7 g/l purified agar (Oxoid)
0.1 mg/l kinetin (Sigma)
pH 5.8.