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Microprojectile-mediated transformation of *Ornithogalum thyrsoides* hybrid A2

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Thesis presented for the degree of
Doctor of Philosophy
in the department of Microbiology,
University of Cape Town

August 1999

Certification of Supervisors

In terms of paragraph 9 of "General regulations for the degree of Ph.D." we, as supervisors of the candidate S.M. de Villiers, certify that we approve the incorporation in this thesis of material that has been submitted for publication.

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Acknowledgements

My sincere thanks go to the following institutions and people:

- The Biotechnology Division of ARC-Roodeplaat, Hadeco, the USDA and UCT for financial support
- Dave Berger - supervisor, fellow scientist, friend and "heavy" - an interesting combination which was an invaluable contribution to this study
- Johan Burger, who initiated this project because he did not want to see the 'tjienkerintjies' die of OrMV
- Professor Jennifer Thomson, who invited me to collaborate with her at UCT on the Taxi technique
- My colleagues at Roodeplaat for many a shared laugh and their willingness to bounce ideas around to solve some "sticky" problems
- Chris Bornman, for providing insights into a world not quite as small as that of DNA
- Kathy Kamo, for enthusiasm, support and friendship during my fellowship at the USDA and since
- My parents, Ben and Elsa Strydom, for always being there and for teaching me perseverance and a love for science
- Etienne, for love, support and patience, especially with my computer moods and -pains. Also, for broadening my horizons and teaching me to dream big.

Abbreviations

ARC	Agricultural Research Council
ARC-Roodeplaat	ARC-Roodeplaat Vegetable and Ornamental Plant Institute
BA	6-benzylaminopurine
bar	phosphinothricin acetyl transferase
BME	ß-mercaptoethanol
bp	base pairs
C	Clone of T1
CaMV35S	cauliflower mosaic virus 35S promoter
CsCl	cesiumchloride
CSPD	disodium3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
CTAB	cetyltrimethylammonium bromide
°C	degrees Celsius
2,4-D	2,4-dichlorophenoxyacetic acid
Dicamba	3,6-dichloro-2-methoxybenzoic acid
DIG	digoxygenin
DNA	deoxyribonucleic acid
dH_2O	distilled water
dNTP	deoxynucleoside 5' triphosphate
dsDNA	double stranded DNA
EDTA	ethylenediaminetetraacetate
EtBr	ethidium bromide
EtOH	ethanol
g	gram
GTC	guanidine thyocyanate
GUS/*uidA*	ß-glucuronidase
h	hour(s)
hpt	hygromycin phosphotransferase (hygromycin resistance)
IAA	indole 3-acetic acid
M	molar
min	minute(s)
MS medium	Murashige and Skoog medium
MW	molecular weight
NAA	α-naphtalene-acetic acid
NaCl	sodium chloride
NaOAc	sodium acetate

NOS	nopaline synthase
NPTII	neomycin phosphotransferase (kanamycin resistance)
NT	NOS terminator
OD	optical density
OrMV	ornithogalum mosaic virus
PAT/pat/bar	phosphinothricin acetyl transferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIG	particle inflow gun
PPT	phosphinothricin
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
sec	second(s)
SSC	saline sodium citrate
ssDNA	single stranded DNA
T	Transgenic plant from the UCT study
TAE	Tris acetate EDTA (buffer)
T-DNA	transfer-DNA
TE	Tris-EDTA (buffer)
Ti-plasmid	Tumour inducing plasmid
TNE	Tris-NaCl-EDTA (buffer)
U	units of enzyme
UCT	University of Cape Town
USDA	United States Department of Agriculture
V	volts
X-Gluc	1-bromo-2-chloro-3-indolyl-ß-glucuronic acid

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Chapter 1

Summary

This study investigated the feasibility of biolistic transformation for *Ornithogalum*. Two PDS 1000/He biolistic guns were used in independent studies, one in 1995 at the Floral and Nursery Plants Research Unit of the National Arboretum of the United States Department of Agriculture (USDA) and the other in 1996 at the Microbiology Department, University of Cape Town (UCT). In the study at the UCT, the Taxi system (Chen *et al.*, 1998) was also used in addition to conventional biolistics to determine which method was most effective in stable transformation studies with *O. thyrsoides* x *O. dubium* hybrid, designated A2.

First, a tissue culture system was developed for A2 that was suitable for transformation studies. Regeneration from leaf explants was optimised in a differential experiment with BA and NAA. MS medium supplemented with 0.5 µM NAA, 9 µM BA and 3% sucrose (Nel, 1981) was the most effective. Callus was identified as the target for transformation. Efficiency of callus initiation from leaf explants was compared on MS medium supplemented with 2,4-D, dicamba, NAA or IAA. Dicamba was the most effective and regeneration of plants from this callus was subsequently optimised.

The origins of plants that regenerated from callus were investigated histologically to determine whether they were single-celled or multicellular. Possible pathways by which regeneration from callus could occur were also examined. It was confirmed that callus tissue consisted predominantly of undifferentiated cells that could differentiate into adventitious buds and shoots. A selection protocol for *Ornithogalum* explant tissues was also established. Lethal concentrations of the selection agent PPT, the active ingredient of the herbicide Ignite®, were determined to be 5 - and 10 µM PPT for leaf explants and callus, respectively.

Second, a transient transformation protocol was optimised for A2 with the GUS gene. Optimised conditions for transient transformation in the two studies were

helium pressures of 5500 to 6200 kPa, gold microprojectiles, 0.25 M osmoticum and CaMV35S promoter.

Stable transformation was attempted in both laboratories using the *pat* herbicide resistance gene. In the first study at the USDA, 137 of the 346 shoots that regenerated on selection medium formed roots. At the UCT, the Taxi method, which entailed complexing single stranded DNA containing the *pat* gene with histone H1 prior to bombardment was also employed. In this study, 129 shoots were obtained with conventional biolistics of which 47 formed roots; with the Taxi, 182 shoots were regenerated of which 74 formed roots.

These putative transgenic plants were propagated *in vitro* and submitted to molecular analyses. Results of PCR analyses of the USDA plants were inconsistent. In contrast, the strongest plant from UCT, designated T1, and five clones propagated from it, did contain the *pat* gene. This single transgenic plant and 13 of the 37 clones derived from it were analysed in further experiments. Southern- and northern blots performed on eight of the clones confirmed integration of the *pat* gene into the genomic DNA and the transcription of RNA. Results obtained with leaves of 13 clones painted with a 3% (v/v) solution of the herbicide Ignite® (30 mM PPT) as well as ELISA results obtained with seven of these clones confirmed expression of the PAT enzyme in tissues of these plants. Finally, greenhouse trials indicated that the transgenic plants were phenotypically normal with regard to leaf- and flower morphology and that leaves of these plants reintroduced into tissue culture, could regenerate plants on medium supplemented with 10 µM PPT.

Chapter 2

General introduction

2.1 *Ornithogalum* - a brief history

Ornithogalum, a genus of about 200 species, is found in Europe, Africa, the Mediterranean and western Asia. Fifty-four species occur in South Africa, including several with commercial potential in the flower trade and some that are poisonous to livestock (Leighton, 1944; Obermeyer, 1978).

The name originates from *ornithogalen*, Greek for bird's milk, which was also a Roman phrase that referred to something wonderful. It was probably used to describe the white flowers of the Mediterranean species, which also occurs in Israel where it was referred to in the Bible as the "star of Bethlehem", a name that is still in use today. The vernacular name, chinkerinchees or tjienkerintjie, imitates the characteristic squeaky sound of the fresh peduncles rubbing against one another (Obermeyer, 1978).

White or creamy coloured cut flowers, mostly selections of *O. thyrsoides*, *O. arabicum*, and *O. saundersiae*, are cultivated in South Africa, Kenya, Israel and the USA (Roos and Pienaar, 1966; Obermeyer, 1978). Their long-lasting freshness makes them ideal for export. Stalks dipped in wax last for weeks while the buds expand gradually. Many more improved lines were developed in breeding programmes in South Africa (Roos and Pienaar, 1966), USA (Griesbach *et al.*, 1993; Griesbach and Meyer, 1998) and Israel (de Hertogh and Gallitano, 1996). However, virus infection and lack of production protocols prevent large-scale commercialisation of improved hybrids (Burger and von Wechmar, 1988; Griesbach *et al.*, 1993; Wanghai and Bock, 1996).

2.2 Economical importance

The Dutch flower- and ornamental plant market was estimated at $ 2.5 billion in 1993 of which 70% was cut flowers. Bulbs comprised 18% of this market (Vereniging van Bloemenveilingen, 1993; Deroles *et al.*, 1997). Although tulips, lilies and gladioli are the most important bulbs, the market share of minor bulbs has increased significantly since 1980 because of the constant demand for novel varieties.

Ornithogalum is a genus in this category and recently gained popularity on international flower markets. On Dutch auctions alone, sales increased from 21 million cut flowers - mostly white - in 1991 to 32.4 million in 1993. During 1993, Israel exported 21 000 orange *O. dubium* flowers to Europe, increasing rapidly to 15 million in 1997/98 (A Cohen, pers. comm.). In South Africa, up to ten tons of flower stems of a white, virus tolerant, *O. thyrsoides* hybrid is exported weekly to Japan (D. Wilson, Hadeco bulb growers, pers. comm.). In addition, millions of bulbs are sold around the globe as "dry sales" for gardening (R Griesbach, USDA, pers. comm.).

Improved selections and hybrids of mostly *O. thyrsoides* and *O. dubium* were obtained from breeding programmes around the world. In South Africa, cut flower varieties with increased flower production, taller and stronger inflorescences, larger- and more flowers and darker colours were selected (Roos and Pienaar, 1966; G Littlejohn, ARC-Roodeplaat, pers. comm.; D. Wilson, Hadeco, pers comm.). At the USDA, hybrids of *O. thyrsoides* and *O. dubium* include cut flower types with orange flowers and pot-plant types with strong inflorescences and increased flower production (Griesbach et al., 1993; Griesbach and Meyer, 1998). In Israel, *O. dubium* hybrids were developed as cut flowers and pot-plants. However, as seed-propagation of hybrids often leads to variations in colour, plant height and flowering time in the progeny, these lines have to be propagated vegetatively for large-scale production (de Hertogh, 1996; de Hertogh and Gallitano, 1996). In isolated cases, hybrids have been developed which propagate homogeneously from seed, but these bulbs can be grown only for a single season before they become infected with OrMV (Asa's bulb growers, Israel and A. Cohen, pers. comm.)

OrMV infection is the most severe constraint on production. All promising hybrids are susceptible to OrMV, which infects rapidly and causes flower stunting and deformation (Smith and Brierly, 1944; Burger and von Wechmar, 1988; Wangai and Bock, 1996). It is readily transmitted by several species of aphids and Wangai and Bock (1996) found that 35% of a virus-free population of *O. thyrsoides* propagated *in vitro* became infected within four months after planting, and 100% within eight months. In the field, stock needs to be replaced every second or third season since OrMV infection results in significant decreases in yield and quality (Cohen et al., 1995). Chemical control is ineffective because aphids that feed on treated plants become agitated and move more frequently between plants, in effect spreading the disease more rapidly. (Harms, 1992) The alternative, virus-free cultivation in insect-proof greenhouses is expensive. Since most *Ornithogalum* hybrids are propagated vegetatively, virus present in source plants are transmitted to all plants derived from them, preventing commercial cultivation.

On the whole, floricultural breeding practices are confounded by several factors, some of which are especially restraining to *Ornithogalum*. The variety of flower crops and number of cultivars per crop is much wider than in food crops and is also changing more rapidly. The diversity in flowers required by the market and short market lifetimes of some cultivars may complicate breeding programmes. Furthermore, long juvenile periods of bulbous flowers like *Ornithogalum* slow down breeding programmes. Augmenting conventional floriculture breeding programmes with molecular genetics may overcome some of these obstacles. Perhaps the greatest possibility for improvement will be the introduction of effective disease and pest resistance genes. In this regard, natural disease resistance is unattainable for many crops but several plant resistance genes have been cloned recently, including one against a virus (Whitman *et al.*, 1994). Furthermore, results obtained in the past decade, showed that plants transformed with viral genes can become resistant to infection by that virus (reviewed by Cuozzo *et al.*, 1988; Beachy, 1997), a strategy which holds much promise for *Ornithogalum*.

2.3 The need for a transformation system for *Ornithogalum*

Industrialised agriculture suffers major losses from diseases, pests and weeds and protection strategies have created a billion-dollar industry for agrochemical companies in a few decades. Agricultural land is treated with pesticides in repeated applications. Industry, farmers and consumers in industrialised countries have become used to and benefit from this practice. More and better chemicals are developed for more applications enabling farmers to increase production and consumers to have a cheap and reliable food supply. Only recently have environmental activists voiced protest against what they perceive as poisoning of natural resources (Harms, 1992; Beachy, 1997; Gelvin, 1998).

In flower crops, the priority of resistance breeding is low compared to breeding for phenotypic and production characteristics. As a result, ornamentals are susceptible to a range of pests and diseases but little flower damage is tolerated by the industry (de Hertogh, 1996). Complete resistance is difficult to achieve but partial resistance, in conjunction with agrochemicals enabled the production of high quality flowers to date. Worrying is the fact that enormous amounts of chemicals are tolerated as the product is not for consumption and residue build-up is of no disadvantage to the final product. These practices have led to the false notion that diseases and pests are not major problems in flower production (RJ Griesbach and A Cohen, pers.comm.).

Pressure to reduce or eliminate the use of agrochemicals in flower production has risen sharply in the past decade. The Netherlands has undertaken to cut pesticide use in greenhouses by 50% by the year 2000. Some companies even withdrew their chemical control agents from segments of the ornamental plant industry. This dwindling availability of chemicals and mounting pressure for less hazardous alternatives for control of ornamental plant pathogens has increased the demand for disease and pest resistant plants. One alternative is to provide plants with an enhanced arsenal of genetic self-defence mechanisms against pathogen attack to reduce the use of pesticides in the long term (Harms, 1992).

All living organisms produce antimicrobial substances and/or other defence responses to survive pathogen attacks. However, modern high-yielding crop

varieties lack much of the natural disease- and pest resistance of old land races or related wild species because during breeding for high yield and other desirable traits, resistance genes were lost. Furthermore, horizontal resistance is conferred by multiple genes for which selection is inefficient and difficult so that resistance genes are often lost because breeders do not make a conscious effort to select for their continued presence (Vanderplank, 1963; Parleviet and Zadoks, 1977; Davis *et al.*, 1990; Harms, 1992; Robinson, 1996).

In the past twenty years, molecular biology has become a tool to merge molecular genetics and plant breeding to improve crops for resistance traits, improved flavour and enhanced processing characteristics. Methods have been developed for the introduction and expression of foreign genes in food crops such as rice (Shimamoto *et al.*, 1989; Pinto *et al.*, 1999), maize (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990), wheat (Vasil *et al.*, 1992), potato (de Block, 1988; Newell *et al.*, 1991) and soybean (Christou *et al.*, 1988). Success is largely due to enrichment of crop gene pools with genes from prokaryotic, viral and non-plant eukaryotic sources, as well as from sexually incompatible plants, which was previously impossible (Christou *et al.*, 1991, Vasil *et al.*, 1992).

However, traditional breeding continues to be the principal source of new cultivars and varieties for the ornamental plant market. Its success is evident in the huge array of products available to the consumer. But it has several limitations, the most obvious being the limited gene pool within a species and the need for successive backcrosses to purify the desired trait away from undesirable traits in the original offspring (Deroles *et al.*, 1997, de Jong, 1999).

Genetic transformation overcomes both these disadvantages. Since the DNA used in transformation can be isolated from any organism, gene pool barriers are eliminated. Transformation involves the transfer of at most a few genes into the host plant, resulting in progeny that is genetically identical to its parents with the addition of one or two characteristics. The desirable horticultural properties of the host plant are preserved and no backcrossing is necessary to purify the desired phenotype (Harms, 1992; Deroles *et al.*, 1997), except in cases such as maize, where "elite" lines are

difficult to transform. For these crops, inbred lines are transformed and the trait is then crossed back into the desired parental lines (Ishida *et al.*, 1996).

2.4 Plant transformation – an overview

Transformation of a plant relies on the transfer of genes to a single plant cell, their stable incorporation into the cell's genome and regeneration of an intact plant from the transformed cell. Genetically engineered plants were first reported in 1984 (de Block *et al.*, 1984; Horsch *et al.*, 1984). Since then more than 120 species in at least 35 families have been transformed including the major cereals, vegetables, ornamental-, medicinal-, and pasture plants, fruit- and other trees (Birch, 1997). Development of transgenic flower crops has lagged behind that of vegetable and grain crops with the exception of petunia, but the attention granted this flower has been almost exclusively as a model system for gene expression and regulation studies, rather than genetic improvement for commercial production. Since 1990, at least 20 more cut flower species have been transformed (Mol *et al.*, 1995; Vainstein and Weiss, 1995), including the monocots tulip (Wilmink *et al.*, 1992), lily (Cohen and Meridith, 1992; Watad *et al.*, 1998) and gladiolus (Kamo *et al.*, 1995).

Genetic transformation has opened new possibilities to the ornamental plant industry, facilitating the development of flowers with novel traits and as an alternative to chemical control (Schell, 1987; Nelson *et al.*, 1988; Gasser and Fraley, 1989; Mol *et al.*, 1995; Vainstein and Weiss, 1995; de Jong, 1999). Meyer *et al.* (1987) first reported manipulation of flower colour using recombinant DNA techniques. Since then, numerous papers have appeared on the molecular manipulation of floral pigmentaion pathways, with both sense- and antisense approaches being successful (Elomaa *et al.*, 1993; Holton and Cornish, 1995 and Vainstein and Weiss, 1995; Tanaka *et al.*, 1998).

Transgenic plants are also used to manipulate gene expression by increasing the amount of a gene product or by targeting expression to unique stages of plant development or organs where it is not normally expressed (Hoffman *et al.*, 1987; Schell, 1987). Gene expression can also be limited with antisense RNA as was done for polygalacturonase production in tomatoes to increase shelf life (van der Krol *et*

al., 1988a; Sheehy *et al.*, 1988; Smith *et al.*, 1988) and with chalcone synthase in petunia to alter flower colour (van der Krol *et al.*, 1988a, 1988b).

Success can be attributed to the development of efficient gene vector systems and plant transformation and regeneration techniques (Cocking and Davey, 1987; Zaitlin and Hull, 1987; Gasser and Fraley, 1989). The two transformation techniques that have found widest application are indirect *Agrobacterium tumefaciens* mediated transformation (reviews by Hooykaas and Schilperoort, 1992; Gelvin, 1998) and direct biolistic or particle gun mediated transformation (Klein *et al.*, 1987; Sanford *et al.*, 1987).

Agrobacterium-mediated gene transfer

A. tumefaciens, a soil-borne plant pathogen, is a natural 'genetic engineer'. It infects plants, mostly dicots, that secrete phenolic compounds at wound sites and transfers a piece of DNA from its tumour inducing (Ti) plasmid to the infected plant. This mechanism has been exploited in plant biotechnology to transfer desirable genes to agronomically important crops. The phenolics act as specific elicitors for T-DNA transfer (Citovsky *et al.*, 1988) and *A. tumefaciens* uses three genetic components for this process - T-DNA, *vir* genes and chromosomal virulence loci - although only the T-DNA is transferred to the plant cell. The *vir* region includes eight gene operons - *virA* to *virH*, of which most contain several genes - encoding *trans*-acting proteins essential for T-DNA transfer. The chromosomal loci code for proteins involved in the binding of *Agrobacterium* to the plant cell during infection (Rogers *et al.*, 1986; Schell, 1987; Zambryski 1988; Hooykaas and Schilperoort, 1992).

The T-DNA, contained on the Ti plasmid, is flanked by 25 bp directly repeated DNA sequences called T-DNA borders. These borders are acted upon by a T-DNA border specific endonuclease encoded by the *virD1* and *virD2* genes (Jasper *et al.*, 1994). It is postulated that a single-stranded DNA molecule, the T-strand, is then unwound from the Ti-plasmid. T-DNA is transferred to the plant as a single-stranded molecule (Tinland *et al.*, 1994), most likely covalently linked to VirD2 protein and perhaps coated by VirE2 protein, a non-specific single-stranded DNA-binding protein also produced by *Agrobacterium*. This single-stranded T-DNA nucleoprotein complex is protected from nuclease attack during transfer. It was also reported that VirD2 is

targeting the T-DNA to the plant cell nucleus and directs integration into the genome, assisted by the plant's DNA repair system (Citovsky *et al.*, 1992; Rossi *et al.*, 1993; Kononov *et al.*, 1997). It was initially thought that integration is precise; only the DNA bordered by 25 base pair direct repeat "integration sequences" is transferred and integrated in the genome and often, only one T-DNA copy is inserted per cell (Zambryski, 1988; Hooykaas and Schilperoort, 1992). However, it was recently found that in up to 75% of plants transformed with *Agrobacterium*, vector backbone sequences outside the T-DNA borders are also integrated into the plant genome (Kononov *et al.*, 1997). It was also found that T-DNA does not always integrate into the plant genome as a simple, unique event but can also integrate as T-DNA multimers which are joined to each other in all possible orientations (de Neve *et al.*, 1997; Gelvin 1998).

As stated before, *Agrobacterium* infects mostly dicotyledonous crops. Protocols are generally simple and efficient and for *Arabidopsis thaliana* it is even possible to transform tissues *in planta* by vacuum infiltration of whole plants (Bechtold *et al.*, 1993) or dipping of developing flower tissues (Clough and Bent, 1998), thereby excluding the need for a tissue culture regeneration protocol. Where ornamental plants are concerned, success with carnation (Lu *et al.*, 1991; Vainstein and Weiss, 1995; Zuker *et al.*, 1995) and rose (Firoozabady *et al.*, 1994) as well as with the monocots tulip (Wilmink *et al.*, 1992), gladiolus (Graves and Goldman, 1987; Kamo, 1997) and lily (Langeveld *et al.*, 1996) has been reported. However, the procedures for monocots yielded small numbers of transgenic plants and many cultivars appeared not to be responsive to *Agrobacterium* infection. Possible reasons are that monocots do not secrete wounding-associated phenolics (Stachel *et al.*, 1985) or exhibit cell proliferation at wound sites (Gheysen *et al.*, 1987). Although *Agrobacterium* mediated transformation is the most popular technique for the transformation of ornamentals (Courtney-Gutterson, 1993; Robinson and Firoozabady, 1993) efficient transformation of monocot flowers such as *Ornithogalum* with this bacterium seems unlikely at present. Recently, super binary vectors, containing additional copies of the *virG* genes were used to successfully transform rice (Hiei *et al.*, 1994), maize (Ishida *et al.*, 1996) and cassava (Li *et al.*, 1996) and holds much promise for other recalcitrant monocot and dicot species.

Direct gene transfer

Direct gene transfer can be applied to any plant species. Techniques include protoplast transformation (Paszkowski *et al.*, 1984; Shillito *et al.*, 1985; Power *et al.*, 1986; Zhang and Wu, 1988; Kartzke *et al.*, 1990) often mediated by the addition of PEG or Mg^{2+}, electroporation (Lurquin, 1997) as well as laser (Guo *et al.*, 1995), silicon carbide fibre (Kaeppler *et al.*, 1992; Serik *et al.*, 1996) and particle gun transformation (Klein *et al.*, 1987; Sanford *et al.*, 1987). Of all these techniques, particle bombardment has been shown to work repeatedly and is relatively inexpensive and straightforward to apply to a range of previously difficult targets. Therefore, where *Agrobacterium*-mediated transformation is not effective, microprojectile bombardment is the preferred method for obtaining transgenic plants (Birch and Bower, 1994).

It was developed by Sanford and co-workers at Cornell University who built a range of devices to accelerate tungsten microprojectiles (1-4µm in diameter) to sufficient velocities (250- to 300 $m.s^{-1}$) to penetrate plant cell walls and membranes. They realised that particle bombardment could be a nearly universal mechanism for transporting substances such as biological stains, proteins (antibodies or enzymes), synthetic macromolecules and genetic material into any living cell, with particular significance for genetic transformation (Klein *et al.*, 1987; Sanford *et al.*, 1987; McCabe *et al.*, 1988).

The inventors named this technique 'biolistics' (Sanford *et al.*, 1987), but it is also referred to as particle bombardment, microprojectile bombardment, particle acceleration, gene gun and particle gun transformation. Microprojectiles are particles of an inert, heavy metal, usually tungsten or gold, and are coated with DNA containing the genes to be transferred. The bombardment process introduces the genetic material into living cells, which survive to express and sometimes incorporate these genes in the chromosomal DNA (Russel *et al.*, 1990; Birch and Bower, 1994). Plants can regenerate from these transformed cells if regenerable tissue was chosen as the target (Vasil and Vasil, 1994), and a single bombardment can yield many transformed cells (Russel *et al.*, 1992).

In particular, the technique has allowed the transformation of crop species and cultivars that are not susceptible to *Agrobacterium* and that cannot be regenerated from protoplasts (Birch and Bower, 1994). DNA has been delivered to protoplasts, cell suspensions, callus tissue, immature embryos, somatic embryos, meristems, microspores, pollen and immature inflorescence tissue (Kartha *et al.*, 1989; Twell *et al.*, 1989; Vasil and Vasil, 1996). Particle bombardment has also facilitated organelle transformation, for example chloroplasts (Boynton *et al.*, 1988; Blowers *et al.*, 1989; Svab *et al.*, 1990; Benediktsson *et al.*, 1995) and mitochondria (Johnston *et al.*, 1988). Apart from plants, bacteria, fungi, algae, animals and yeast have also been transformed (Sanford *et al.*, 1991; Birch and Bower, 1994).

Particle bombardment devices

Several devices have been designed to accelerate microprojectiles using either electric discharge (Christou *et al.*, 1988), gunpowder or compressed helium propulsion systems (Sanford *et al.* 1987; Oard *et al.*, 1990). Helium is preferred as it is clean, controllable, safe and readily available. It expands much faster than air, therefore, a given pressure imparts a higher velocity to the microprojectiles (Deroles *et al.*, 1997). Two helium guns are widely in use; the particle inflow gun (PIG)(Finer *et al.*, 1992; Bond, 1993; Vain *et al.*, 1993) and the PDS 1000/He device (reviewed by Kikkert, 1993). The PIG is cheap and easy to build but is not commercially available unlike the PDS 1000/He device (DuPont Biolistic®). In addition, reports showed that the latter device could introduce DNA into bacterial, fungal, plant and animal targets (Klein *et al.*, 1992).

Particle bombardment is notorious for variability of results between consecutive bombardments under 'identical' conditions. This problem can be overcome if high transient expression frequencies of introduced DNA can be obtained routinely in potentially regenerable cells (preferably several hundred events per bombardment). Sufficient supplies of target tissue are also essential as only a small proportion of penetrated cells is stably transformed and able to regenerate plants (Klein *et al.*, 1992). In this regard, the PDS 1000/He device has been shown by hundreds of users to be effective (Birch and Bower, 1994; Wong, 1994).

Transient expression

Plant transformation, whether indirectly or directly with particle bombardment, delivers additional genes into a cell's chromosome. If the target cell survives this process, the transferred genes can be transcribed and translated into proteins, whether the gene was inserted in the genomic DNA or not. Initially it is impossible to differentiate between expression of proteins from transgenes inserted into the genome, which is the ideal situation and those that are transcribed from extra-chromosomal elements. Transgene expression observed within days after bombardment is termed transient transformation, because, if the gene was not inserted into the chromosomal DNA, expression is detectable for only a few days before it is diluted and disappears with subsequent cell divisions. Therefore transient expression does not indicate the frequency of chromosomal integration, but it confirms that the transgene has crossed the cell wall, that the cell survived the transformation process and that the transgenic trait is expressed. In most biolistic plant transformation studies, transient expression of a reporter gene such as *uidA*, which codes for GUS, are used to optimise bombardment conditions in regenerable tissues (Jefferson *et al.*, 1987). GUS enzyme activity is visualised as blue spots after histochemical staining with X-Gluc; each spot represents a transformed cell or cluster of cells. Other reporter genes that are often used are chloramphenicol acetyl transferase (Kartha *et al.*, 1989), luciferase (Ow *et al*, 1986) and GFP (Hasselhof and Amos, 1995; Hasselhof *et al.*, 1997) or gene fusions combining GUS and GFP (Quaedvlieg *et al.*, 1998).

According to Klein *et al.* (1992), four key factors interact to affect the frequency of transiently expressing cells in the bombarded tissue. The composition and size of the microprojectiles are important. In cases where tungsten is cytotoxic, gold is used as microprojectiles. Larger particles reach higher velocities, but also cause more damage to small cells. The efficiency of DNA attachment to the microprojectiles prior to bombardment will determine how many intact copies of the transgene reach the target cell. The impact velocity of the microprojectile/DNA complex has to be sufficient to penetrate cell walls without killing the cells and is determined by the strength of the propulsion force, the distance the microprojectiles travel, and the degree of vacuum in the chamber. Finally, the amount of tissue damage suffered on bombardment has to be minimised to enable the small percentage of transformed

cells to survive and regenerate into plants. Some general principles apply to the selection of a starting range for each of these factors and it is not unusual to increase transient expression frequencies by one or two orders of magnitude from poor initial results as these parameters are optimised for a particular system (Sanford *et al.*, 1991; Klein *et al.*, 1992).

The Taxi transformation system

One drawback of particle gun transformation is that the process as such does not direct chromosomal integration of transgenes, as *Agrobacterium* does. Generally, plasmids without nuclear targeting signals are used as transgene vectors and are not protected by DNA-binding proteins. Random linearisation during bombardment may disrupt chromosomal integration but this problem can be alleviated by precise linearisation of the transformation vector with restriction enzymes prior to bombardment. In addition, Rondenburg *et al.* (1989) showed that genomic integration of dsDNA is 3-10 fold less efficient than that of ssDNA in *Nicotiana* protoplasts and Tinland *et al.* (1994) further showed that T-DNA entering the plant nucleus is predominantly single stranded. When dsDNA does integrate, multiple copy insertions and tandem repeats of transgenes often occur. This is less often the case with *Agrobacterium*-mediated transformation (Gelvin, 1998).

Chen *et al.* (1998) circumvented the disadvantages of particle gun transformation with naked plasmid DNA by developing a system that imitated *Agrobacterium* mediated transformation with an artificial T-DNA complex, termed the Taxi. They exploited the findings of Hugues *et al.* (1965) who reported that basic polypeptides, such as histones, increased cell membrane permeability and was absorbed 3000 times faster than serum albumin. If serum albumin was mixed with histones, uptake increased 10- to 50-fold (Drew et al., 1970) and the authors concluded that histone H1 facilitated entry of biological macromolecules into eukaryotic cells. These findings are supported by the fact that histone H1 is a chromosomal DNA-binding protein and as such contains amino acid targeting sequences for entry into the cell nucleus (Dingwall and Allan, 1984; Shanon and Wells, 1987). As a result, Chen *et al.* (1998) postulated that histone H1 could function as ssDNA-binding protein and also facilitate entry of the bound DNA into the nucleus. In addition, the protein was shown to have recombinase activity, catalysing ATP independent DNA strand transfer and

promoting integration of ssDNA into viral chromosomal DNA (Sobczak and Duguet, 1988; Kawasaki *et al.*, 1989). As a result, histone H1 from sea urchin sperm was chosen to function like VirD2 and VirE proteins in the Taxi construction.

Linearised ssDNA was obtained by heat denaturation of linearised plasmid DNA containing both a selectable marker- and reporter gene. Reannealing of complementary strands was prevented by complexing with the DNA-binding, nuclear targeting protein, histone H1 from sea urchin sperm purified at the University of Cape Town (von Holt *et al.*, 1989). This complex also protected the ssDNA from nuclease attack and directed entry into the nucleus, similar to the VirD2 and VirE proteins of *A. tumefaciens*.

Chen *et al.* (1998) transformed *Digitaria sanguinalis* combining biolistic transformation with the Taxi. Genomic integration and transgene expression in the R1 and R2 generations of transformed plants demonstrated that the Taxi can improve direct gene transfer to cereals, bypassing complications posed by the host range specificity of *Agrobacterium*.

2.5 Isolation of transgenic plants

Regardless of the technique employed, a mixture of transformed and non-transformed cells is present after transformation. The transformed cells can be selected from this mixture with a plant selectable marker gene or visualised with a reporter enzyme system (Rogers *et al.*, 1986; Rothstein *et al.*, 1987; Schell, 1987). Most techniques rely on selectable markers that will allow only transformed tissue to grow on media supplemented with the selection agent. The most widely used selectable marker genes for plants are *nptII*, which confers resistance to kanamycin (Hauptmann *et al.*, 1988), *hpt* for hygromycin B resistance (Rothstein *et al.*, 1987) and *bar* or *pat* which detoxifies the herbicide phosphinothricin or PPT (de Block *et al.*, 1987). In this study the *pat* gene was used, which codes for the phosphinothricin acetyl transferase enzyme. PPT inhibits glutamine synthetase, an enzyme which catalyses the synthesis of glutamine from glutamate and ammonia. This reaction detoxifies excess ammonia released by nitrate reduction, amino acid degradation and photorespiration. Inhibition of glutamine synthetase by PPT causes

accumulation of ammonia that leads to cell death (de Block *et al.*, 1987). Plant tissues transformed with the *pat* and *bar* genes are resistant to PPT as well as to herbicides containing this compound as active ingredient.

Regeneration of plants on selective medium is not conclusive evidence for successful transformation. Phenotypic and molecular analyses of antibiotic-resistant plants are necessary to determine if the transferred gene is present, if it is integrated into the genomic DNA and the number of copies present. PCR (Mullis and Faloona, 1987) and Southern blotting (Southern, 1975; Sambrook *et al.*, 1989) are widely used for this purpose. PCR is the easiest method to verify the presence of the gene in a plant but does not prove that the gene is present in every cell or that it is integrated in the genomic DNA (Schaff, 1991).

Only positive genomic Southern blots confirm that the transgenic plant has the transferred gene incorporated into its genome (Schaff, 1991). Southern blot analysis entails extracting the genomic DNA from the plant and subjecting it to gel electrophoresis, either undigested or digested with restriction enzymes. DNA fragments are separated by size, immobilised on a nylon membrane by blotting, and hybridised with a radioactively labelled DNA probe, which is prepared from a sample of the DNA used for transformation. Results are visualised by autoradiography. A Southern blot with undigested DNA will show whether the gene has been incorporated into the genome of the plant or if it remains as an extrachromosomal element. Positive Southern blots with restriction enzyme-digested genomic DNA enables estimation of the number of insertion events and copies of the transferred DNA into the plant's genome (Schaff, 1991). In some cases, restriction digested DNA alone may not be sufficient to determine the copy number if tandem integration of the gene at a single locus had occurred and in such cases densitometric scanning with known copy number controls may be necessary to elucidate the number of integrations (Birch, 1997).

Plants testing positive in Southern blot analyses are transgenic but they do not always exhibit the transgenic trait. An error can occur at various points in the pathway from transcription to expression of the transgenic trait as a functional protein. The gene may be integrated in the DNA but not be transcribed into RNA or

the gene product. If the gene is a selectable marker gene such as *pat*, *nptII* or *hpt*, such plants usually die during selection. However, if a second trait, such as a disease resistance gene, was inserted along with the selectable marker gene, plants that contain the latter gene, may die because the selectable marker gene is not expressed. Northern blot or reverse transcription PCR (RT-PCR) verify that RNA is transcribed from the transgene.

Northern blots are more informative than RT-PCR as they provide quantitative information on the activity of the promoter that drives transcription from the gene as well as on the level of transcription. Both techniques provide information on whether transcription is constitutive, tissue specific or does not occur at all (Schaff, 1991). RT-PCR requires primers directed against the transgene sequence and reverse transcriptase polymerase enzyme to synthesise a double-stranded template from the target RNA that is transcribed from the transgene, which is subsequently amplified by PCR (Colonna-Romano *et al.*, 1998).

A second possible aberration from successful transformation is that RNA is present but not translated into protein. In this case, confirmation of the presence of the protein product, achieved through western blot, ELISA or an appropriate enzyme assay for the transgene product, will illuminate such deficiencies. The first two techniques require antibodies directed against the transgene protein product to verify that it is synthesised and quantify the amount synthesised. If the protein is an enzyme, an assay will confirm the integrity, correct conformation and activity of the transgenic protein (Schaff, 1991). Once the transgenic nature of the plants is verified, they are evaluated in greenhouse and field tests for inheritance, stability and breeding behaviour of the trait (Schaff, 1991).

It is therefore obvious that transgenic plants need to be tested at all levels - DNA, RNA and protein - for the presence and correct expression of the transferred trait. It is especially important to ensure that the transgenic phenotype is expressed under cultivation conditions. Therefore, transgenic plants have to be analysed in a greenhouse and in field trials to confirm the correct phenotype and expression of the transferred trait under these conditions. For *Ornithogalum* this entails confirmation of normal phenotype and flower morphology. If the target crop is propagated through

seed, additional genetic analysis is crucial to determine inheritance patterns and stability of the transgenic trait.

2.6 Aims of the project

To date, no reports have been published on *Ornithogalum* transformation and the aim of this study was to determine if *O. thyrsoides* hybrid A2 was amenable to biolistic transformation. To this end, studies were undertaken to find tissues that were suitable explants for gene transfer, to optimise bombardment conditions, to bombard explant tissues for stable transformation with the *pat* gene, to regenerate plants on selection medium and to prove that regenerated plants were transgenic. As a commercial particle gun was not available at ARC-Roodeplaat, two different PDS 1000/He biolistic guns were used in two independent studies. In one study, the Taxi system was compared to conventional biolistic transformation. The aim was to stably transform *O. thyrsoides* hybrid A2 with the *pat* gene and to determine if the Taxi system had an advantage above conventional biolistics.

Plants that regenerated from bombarded tissues were propagated and analysed for the presence and expression of the transgene with PCR, Southern blots, northern blots, ELISA, leaf assays and greenhouse trials.

At the beginning of each chapter in this thesis, there is a brief literature review pertaining to the topic of the chapter. Chapter 3 concerns the development of a tissue culture protocol for *Ornithogalum thyrsoides* hybrid A2 that is suitable for transformation studies. Chapter 4 entails optimisation of transient transformation and regeneration of plants on selection medium and finally, Chapter 5 covers the molecular analysis and greenhouse trials of putative transgenic plants to verify that the transgene was present and expressed.

Chapter 3

Development of a tissue culture protocol for transformation

3.1 Abstract

A tissue culture system was developed for *Ornithogalum thyrsoides* that could be used in transformation studies. Regeneration from leaf explants was optimised in a differential experiment with BA and NAA. MS medium (Murashige and Skoog, 1962) supplemented with 0.5 µM NAA and 9 µM BA (Nel, 1981) was the most effective.

Callus was identified as the target for transformation. This study compared the efficiency of callus initiation from leaf explants cultured either on 2,4-D, dicamba, NAA or IAA. Dicamba was the most efficient and regeneration of plants from this callus was optimised.

Origins of the regenerated plants, whether single-celled or multicellular, were investigated histologically and possible pathways by which regeneration from leaf explants and callus occurred, were examined. It was confirmed that callus tissue consisted predominantly of undifferentiated cells that could develop into adventitious buds and shoots.

Finally, a selection protocol for transformed tissues was established. Lethal concentrations of the selection agent PPT, the active ingredient of the herbicide Ignite® (ammonium-DL-homoalanin-4-yl methyl phosphate) were determined for callus- and leaf explants. Concentrations of 5 - and 10 µM PPT were lethal for leaf explants and callus, respectively.

3.2 Introduction

Ornithogalum thyrsoides A2, the subject of this study, is an *O. thyrsoides* x *O dubium* hybrid with commercial potential as a cut flower obtained from a collaborative breeding programme initiated in 1963 between ARC-Roodeplaat and the University of Stellenbosch (Obermeyer, 1978). Like most *Ornithogalum* hybrids, A2 is susceptible to ornithogalum mosaic virus, which hampers cultivation of improved hybrids (Burger and von Wechmar, 1988). Therefore, a system that could transfer novel characteristics such as resistance to viruses, bacteria or fungi, different flower colour and fragrance to *Ornithogalum* (Elomaa *et al.*, 1993; Dudareva *et al.*, 1996), would greatly benefit commercial production of this ornamental plant.

Most plant transformation systems require an efficient tissue culture protocol, to provide target tissue for transformation and regeneration from transformed cells (Vasil and Vasil, 1996). *Ornithogalum* spp. are relatively easy to culture *in vitro*, as reported for *O. thyrsoides* (Hussey, 1976; Klesser and Nel, 1976; Nel 1981; Chung *et al.*, 1980), *O. dubium* and O. *lacteum* (D Wilson, Hadeco, South Africa, pers comm) and *O. umbellatum* (Nayak and Sen, 1995). The first protocols were reported independently by Hussey (1976) and Klesser and Nel (1976). Hussey cultured stem, leaf, ovary, sepal or bulb scale tissues on Murashige and Skoog (1962) basal medium, whereas Klesser and Nel cultured bulb scales, leaves and flower stems on Sheridan's medium (Sheridan, 1968) with BA (9 μM) and NAA (0.5 to 5 μM). Subsequently, Nel (1981) developed an improved protocol for mass propagation of *O. thyrsoides* from leaf explants using 9 μM BA and 0.5 μM NAA in MS medium.

In general, regenerable, undifferentiated tissues such as callus and cell suspensions are used for plant transformation, which are subsequently induced to regenerate plants incorporating the added trait (Birch and Bower, 1994; Luthra *et al.*, 1997). Auxins such as 2,4-D, dicamba and NAA generally promote callus formation. Hussey (1976) first reported initiation of callus from stems and leaves on MS medium supplemented with IAA, NAA or 2,4-D. Slabbert (pers. comm.) confirmed that *O. thyrsoides* hybrids developed at ARC-Roodeplaat, proliferated callus from leaf explants cultured on MS medium supplemented with 2,4-D. In this study the

influence of 2,4-D, dicamba, NAA and IAA to initiate callus suitable for transformation studies, was compared.

In addition to callus initiation, regeneration from transformed cells was essential. Therefore, callus was cultured on MS medium supplemented with various combinations of BA and kinetin to identify the most effective cytokinin combination for regeneration.

Three observed and two potential pathways that were postulated by which *Ornithogalum* plants may arise from leaf explants cultured *in vitro* is summarised in Figure 1. Adventitious buds, shoots, bulbils and subsequently plants can be induced either indirectly (pathway A) following the initiation and proliferation of callus or directly (pathway B) without an intermediate callus phase. In both cases, nodules of meristematic tissue are the origin of these differentiated structures. A histological study was conducted to support this hypothesis and investigated the origin of plants, organs and meristematic tissues that differentiated from leaf explants and callus that was cultured on regeneration medium. The structures predicted in Figure 1 to occur during regeneration are discussed shortly:

- *Meristemoid*. The concept of the meristemoid was introduced by Bünning (1952) to describe the localised meristematic activity of specialised cells such as the cells comprising the stomatal complex of stoma mother cells, guard cells and subsidiary cells in the epidermis (Esau, 1977). As the epidermis may retain its potential for growth for a long time, adventitious buds and embryos can arise from it, as was previously shown (Gulline, 1960; Konar and Nataraja, 1965).
- *Adventitious shoot meristems*. Regeneration of *Ornithogalum* from adventitious buds on leaf explants has been reported by Hussey (1976), Klesser and Nel (1976), Nel (1981), Griesbach *et al.* (1993) and Nayak and Sen (1995). In *Lachenalia,* like *Ornithogalum* also a genus in the Hyacinthaceae, Niederwieser and van Staden (1990) showed that adventitious buds arose from guard-cell mother cells. Under certain culture conditions, these cells form meristemoids, which can give rise to adventitious buds, as reported for radiata pine (Yeung *et al.*, 1981) and Norway spruce (Jansson and Bornman, 1981) or to somatic embryos, as was shown for both white and black spruce (Lelu and Bornman, 1990).

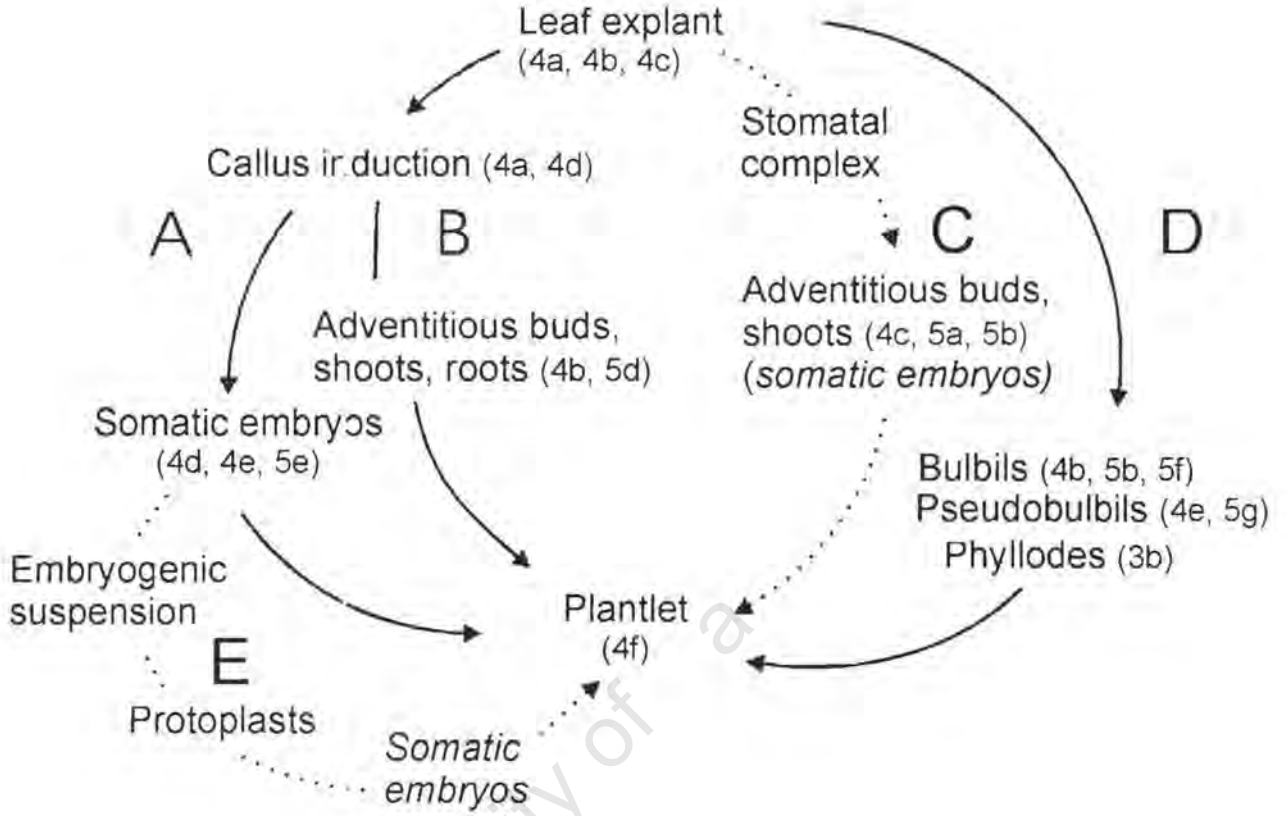


Figure 1 Established and potential pathways for the regeneration *in vitro* of *Ornithogalum thyrsoides* from leaf explants. Parentheses refer to Figures 4 and 5 where anatomical aspects of various developmental events are shown. Bold lines (A-C) indicate verifiable pathways observed in this study, whereas the dashed lines show potential pathways for the regeneration of somatic embryos (D) and non-chimeric plants originating from single cells (E). Italics (*somatic embryos*) serve to indicate that this observation is preliminary.

- *Globular tissue systems*. Leaf explants of many species, including those in the Pinaceae (Bornman, 1985) and the Liliaceae, the family to which *Ornithogalum* formerly belonged, often give rise to globular structures when cultured *in vitro*. Some of these structures appear as bulbils, which may develop into rooted plants. Somatic embryos are also a form of globular tissue, and may differentiate either directly or indirectly following an intermediate callus phase.
- *Callus*. Leaf explants can be induced to proliferate callus, mostly from wounded surfaces. This callus can be separated from the explant and subcultured on an appropriate medium for the initiation of adventitious buds and roots, and possibly also of globular tissues.

This study aimed to determine which of the postulated pathways is applicable when *Ornithogalum* tissues are cultured and plants regenerated *in vitro*.

Finally, a selection protocol was optimised to enable regeneration from transformed tissues in preference to non-transformed cells. PPT, often used in plant transformation protocols (De Block *et al.*, 1987; Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990) was chosen as the selection agent. Leaf explants and callus were cultured on regeneration medium supplemented with various concentrations of PPT (0 – 30 µM).

3.3 Materials and Methods

3.3.1 Regeneration from leaf explants

Leaf explants (10 mm x 10 mm) were cut from virus-free, glasshouse-grown plants of *Ornithogalum thyrsoides* A2. These explants were placed, abaxial surfaces down, on MS medium (Murashige and Skoog, 1962) containing different amounts of BA and NAA (0 or 0.5 µM NAA; 0, 2 or 9 µM BA as well as combinations of the two), 90 mM sucrose and solidified with 8% (w/v) Difco-Bacto agar. Each treatment contained five replicates of ten explants. Tissues were cultured at 26 °C under a 16 h photoperiod and subcultured to fresh medium after four weeks. Shoots that regenerated were allowed to root on MS medium lacking plant growth regulators. Rooted plants, which were used as explants in all subsequent experiments, were maintained on MS medium and subcultured every 12 weeks.

3.3.2 Callus proliferation

Leaves of plants cultured *in vitro* were cut into 40 mm lengths and shallow transverse incisions were made to create wound sites for cell proliferation. These explants were cultured on either 2,4-D (9 µM), dicamba (9 µM), IAA (46 µM) or NAA (27 µM). The medium also included folate (1 µM) biotin (0.2 µM) and sucrose (90 mM). Cultures were kept in the dark at 26°C and subcultured after four weeks. Each treatment contained five replicates of five explants each. Six- to eight-week old callus was used in subsequent regeneration experiments.

3.3.3 Regeneration from callus

Regeneration from callus was optimised in a differential treatment with BA and kinetin (MS medium plus 0, 2 or 4.5 µM kinetin; 0, 2, 4.5 or 9 µM BA as well as combinations thereof). Six- to eight-week-old callus was carefully removed from leaf explants with the blunt side of a scalpel blade. All leaf material was excluded to ensure that regeneration occurred from the undifferentiated cell mass. Explants, initiated on either dicamba or NAA, consisted of 10 mm diameter clusters of callus cells. Each treatment contained four replicates of five explants. The cultures were transferred to fresh medium every four weeks and shoots that developed were transferred to basal MS medium for root initiation.

3.3.4 Histology of regeneration

Organogenesis was studied by fixing and embedding callus tissue, leaf disks and various types of differentiating and differentiated organs and tissue systems. Tissues were photographed under a stereo microscope and sectioned in 5 mm x 5 mm pieces prior to embedding. Plant tissues were fixed overnight in 4% buffered (phosphate, 0.05 M, pH 7.0) glutaraldehyde, washed (3 x 20 min) with phosphate buffer (0.05 M, pH 7.4) and dehydrated sequentially with 2-methoxyethanol (2 x 1 h and 1 x 30 min), absolute ethanol (3 x 20 min), propanol (3 x 20 min) and n-butanol (3 x 20 min). Tissues were infiltrated overnight with two changes of resin, embedded in fresh glycolmethacrylate (Kulzer Histo-Technik®, Heraeus Kulzer, Friederichsdorf, Germany) and allowed to harden for at least 48 h prior to sectioning (Feder and O'Brien, 1968). Sections of 1 – 5 µm were cut with glass knives using a Reichert-Jung ultramicrotome (model 2040) and stained with 0.05% toluidine blue in water (Feder and O'Brien, 1964).

3.3.5 Optimisation of a selection protocol

Leaf pieces and 10 mm aggregates of six- to eight-week old callus cells were analysed in two separate experiments, each consisting of at least two replicates containing five explants that were cultured on regeneration medium supplemented with filter-sterilised Ignite® (Agrevo, Frankfurt, Germany; active ingredient: 1M PPT). The concentrations of PPT tested were 0, 2.5, 5, 7.5, 10, 15, 20, 25 and 50 µM, respectively. Tissues were subcultured after four weeks and evaluated after eight weeks.

3.4 Results

3.4.1 Regeneration from leaf explants

The results presented in Table 1 were confirmed by a second similar experiment. Adventitious bulbs and/or shoots regenerated within three to six weeks predominantly from the cut edges and adaxial surfaces of 56- to 92% of the explants, depending on the medium (Table 1 and Figures 2a and 4b). In a few cases, regeneration from the intact adaxial epidermal were also observed, as illustrated in Figure 4c. The total number of shoots obtained from all explants in each treatment is also presented in Table 1.

3.4.2 Callus proliferation

Friable callus with no tracheids or nodules of vascular elements (Figure 4d left panel) was obtained from wound edges of approximately 90% of leaf explants (Figure 4a) and proliferated on callus induction medium containing either 2,4-D, dicamba or NAA (Table 2; Figure 2b). Proliferating callus often enveloped the leaf explants (Figure 4d). No visible difference was observed between the type or amount of callus initiated on either NAA, 2,4-D or dicamba although roots formed in combination with callus from 40% of explants cultured on NAA medium after four to eight weeks. Shoots and roots formed more often than callus from 90% of explants on IAA medium. Table 2 represents the predominant reaction of each explant, although on some explants, callus proliferated in combination with roots and/or shoots.

Table 1 Reaction of leaf explants cultured on various regeneration media for six weeks.

Type of reaction	No. of leaf explants cultured on regeneration media containing BA and NAA at concentrations (μM) of					
BA:	0	0	2	2	9	9
NAA:	0	0.5	0	0.5	0	0.5
No visible reaction	8	2	18	1	11	2
Root formation	0	19	4	9	0	3
Green callus and shoots	42	29	28	40	39	45
Total no. of shoots obtained	93	151	73	342	209	290

Table 2 Reaction of leaf explants cultured on various callus induction media for eight weeks

Type of reaction	Number of leaf explants cultured on callus induction media containing:			
	9μM 2,4-D	9μM dicamba	45μM NAA	27μM IAA
No visible reaction	2	0	2	2
Root/shoot formation	2	2	9	23
Callus proliferation	21	23	14	0

Figure 2a Differential experiment illustrating regeneration from leaf explants cultured for 9 weeks on MS medium supplemented with (clockwise, from top left): no growth regulators; 4.5 µM BA; 9 µM BA; 0.5 µM NAA + 9 µM BA; 0.5 µM NAA + 4.5 µM BA; 0.5 µM NAA.

Figure 2b Differential treatment illustrating callus proliferation from leaf explants cultured for 9 weeks on callus induction medium supplemented with (clockwise, from top left): 9 µM 2,4-D; 9 µM dicamba; 46 µM IAA; 27 µM NAA.

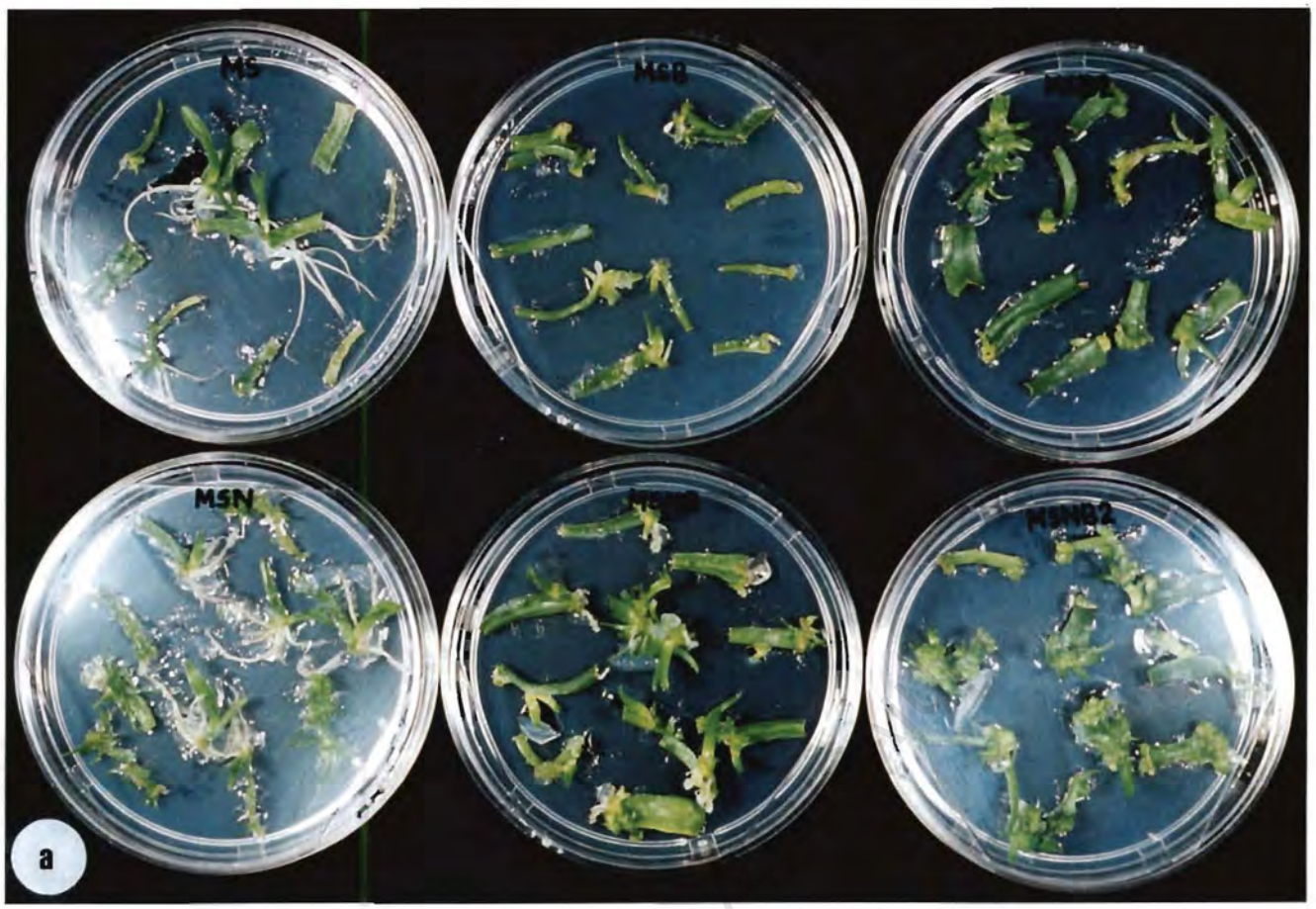


Figure 2

Table 3 Reaction of callus that was initiated on dicamba and cultured on regeneration medium for nine weeks

Type of reaction	No. of explants cultured on regeneration medium containing BA and kinetin at concentrations (μM) of											
BA	0	2	4.5	9	0	2	4.5	9	0	2	4.5	9
kin	0	0	0	0	2	2	2	2	4.5	4.5	4.5	4.5
No visible reaction	1	0	0	1	0	0	0	2	3	0	2	0
Root formation	10	8	9	11	14	120	8	17	14	15	7	12
Green centres	1	1	0	0	0	0	1	0	0	4	4	0
Shoot formation	4	11	9	8	5	7	11	1	3	1	7	6
Total no. explants[a]	16	20	18	20	19	19	20	20	20	20	20	18

[a]A total of less than 20 indicates that some explants were contaminated and discarded

Table 4 Reaction of callus that was initiated on NAA and cultured on regeneration medium for nine weeks

Type of reaction	No. of explants cultured on regeneration medium containing BA and kinetin at concentrations (μM) of											
BA	0	2	4.5	9	0	2	4.5	9	0	2	4.5	9
kin	0	0	0	0	2	2	2	2	4.5	4.5	4.5	4.5
No visible reaction	2	1	0	1	1	0	2	4	6	3	1	1
Root formation	14	4	7	6	5	5	13	9	6	6	6	7
Green centres	0	1	1	0	3	1	1	1	1	2	3	4
Shoot formation	4	9	12	6	7	14	4	6	5	9	9	8
Total no. explants[a]	20	15	20	13	16	20	20	20	18	20	19	20

[a]A total of less than 20 indicates that some explants were contaminated and discarded

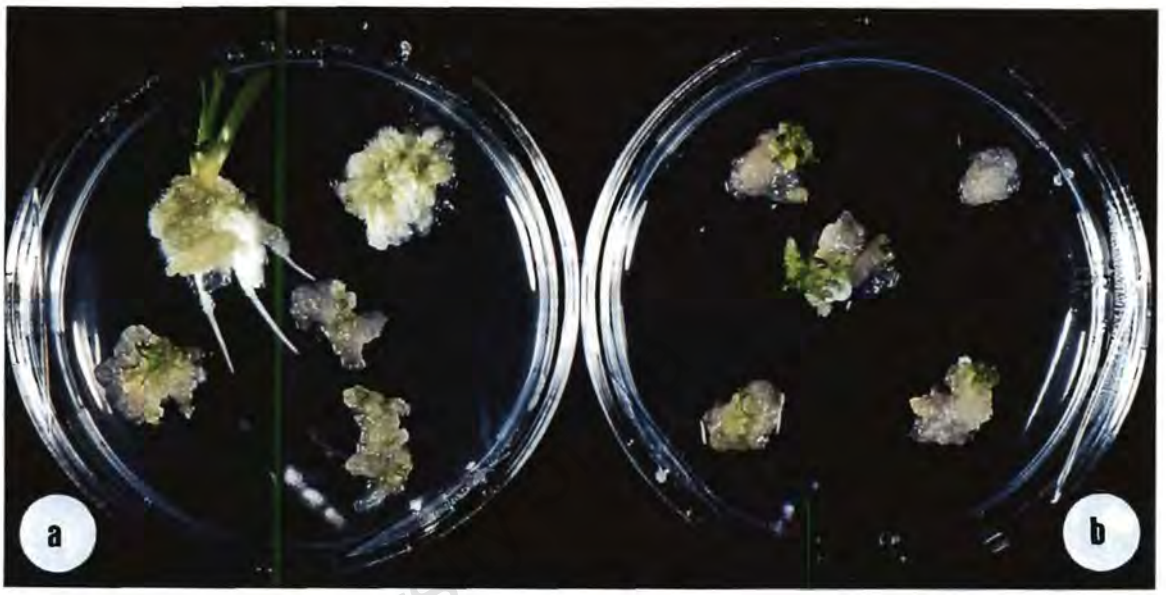


Figure 3 Regeneration of plants from callus obtained from leaf explants cultured on callus induction medium for nine weeks. (a) Callus initiated on NAA and cultured on MS medium containing 2 µM BA and 2 µM kinetin and (b) callus initiated on dicamba and cultured on MS medium containing 4.5 µM BA and 2 µM kinetin.

3.4.3 Regeneration from callus

The results for callus initiated on dicamba are presented in Table 3 and those for callus initiated on NAA are presented in Table 4. Examples of the frequency of regeneration obtained from callus initiated on NAA and dicamba are illustrated in Figure 3.

Plants regenerated from 55% of callus aggregates obtained from medium containing dicamba that were cultured on MS medium supplemented with either 2 µM BA or a combination of 4.5 µM BA and 2 µM kinetin. However, a larger number of more vigorous plants were obtained on the latter medium. For callus tissue initiated on NAA, 70% of explants cultured on MS medium supplemented with 2 µM BA and 2 µM kinetin regenerated plants. In general, more shoots were obtained from callus explants initiated with dicamba compared to those from NAA-containing medium.

3.4.4 Histology of regeneration

Leaf explants wounded on the adaxial surface (Figures 4a and b), give a typical wound response where a cicatrice forms, followed by callus proliferation from cells near the wound surface. In contrast, an explant (Figure 4c) with an intact epidermis may produce adventitious shoots as a result of meristematic activity in a stomatal complex (Figure 5a). This may lead to the initiation of either bud or bulbil primordia (Figure 5b) which can develop into shoots (Figure 4c).

Globular structures (Figures 4b, 5b, 5f and 5g) and leaf-like protuberances (Figures 4b, 5b) differentiated from intact and wounded leaf explants placed on regeneration medium (Figure 2a), from leaf explants cultured on callus initiation medium (Figures 4e left panel, 5g) and from callus cultured on regeneration medium (Figures 4d, 4f, 5d, 5f). Somatic embryos were also initiated, presumably from meristemoids induced in the callus (Figures 4e right panel, 5e).

Figure 4 Macroscopic developmental events in the culture of *O. thyrsoides* leaf explant and callus tissues: (**a**) Leaf explant illustrating the development of a cicatrice at the wound edge and callus tissue inside the wound orifice; (**b**) Phyllode, adventitious bud and shoot formation at wound sites as well as on the adaxial surface of a leaf explant; (**c**) Shoots that developed from stomatal complexes on the intact adaxial surface of a leaf explant; (**d**) Callus tissue that proliferated from a leaf explant which contains undifferentiated cells (left) but can be induced to differentiate globular structures such as bulbils, pseudobulbils and somatic embryos (right); (**e**) Globular structures that were observed on leaf explants and in callus which can develop into either bulbils, pseudobulbils or somatic embryos; (**f**) Organised structures (bulbils, roots, shoots and plants) that differentiated from callus tissue. All bars represent 5 mm.

Figure 5 (Overleaf) Light micrographs of glutaraldehyde-fixed and glycolmethacrylate-embedded tissues of *O. thyrsoides*. The sections (1 – 4 µm thick) were stained with 0.05% toluidine blue: (**a**, **b**) Initiation of meristematic activity from stomatal complexes (bars represent 25 µm); (**c**) Meristematic activity amidst undifferentiated callus cells (bar represents 50 µm); (**d**) Development of shoots and meristemoids on the edge of undifferentiated callus tissue (bar represents 100 µm); (**e**) Longitudinal section through a somatic embryo, illustrating the radicle- and apical meristems connected by vascular tissue along the central axis (bar represents 100 µm); (**f**) Transverse section of a developing bulbil, with the apical meristem in the centre surrounded by the cotyledon (bar represents 50 µm); (**g**) Transverse section through a pseudobulbil, illustrating the absence of differentiation of specific organs (bar represents 50 µm).

Figure 6 (Second overleaf) Effect of the selection agent PPT on (**a**) leaf and (**b**) callus explants after four weeks in culture. Concentrations tested were 0 µM (0 mg/l), 2.5 µM (0.5 mg/l), 5 µM (1 mg/l), 7.5 µM (1.5 mg/l), 10 µM (2 mg/l), 15 µM (3 mg/l), 25 µM (5 mg/l) and 50 µM (10 mg/l) PPT.

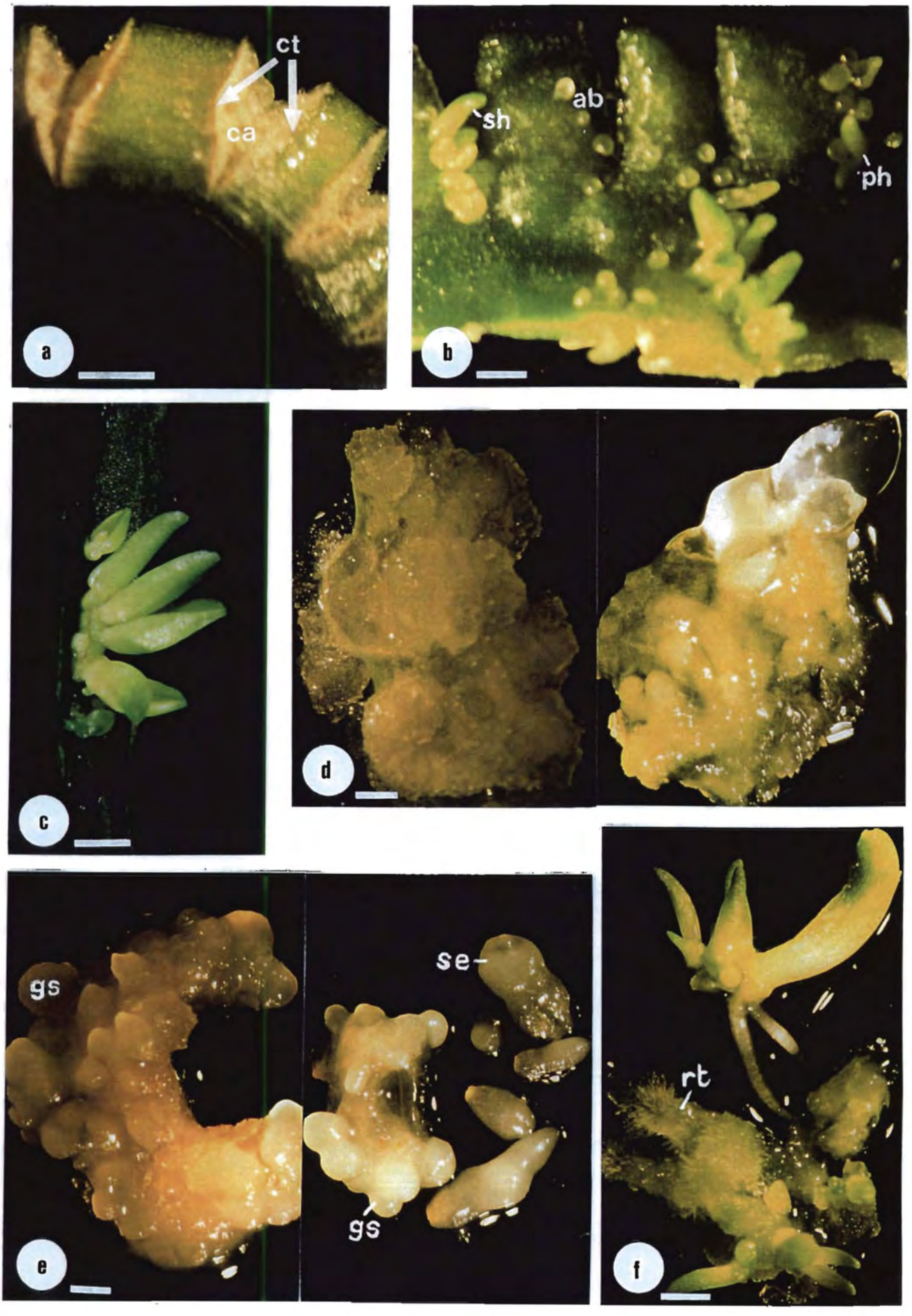


Figure 4

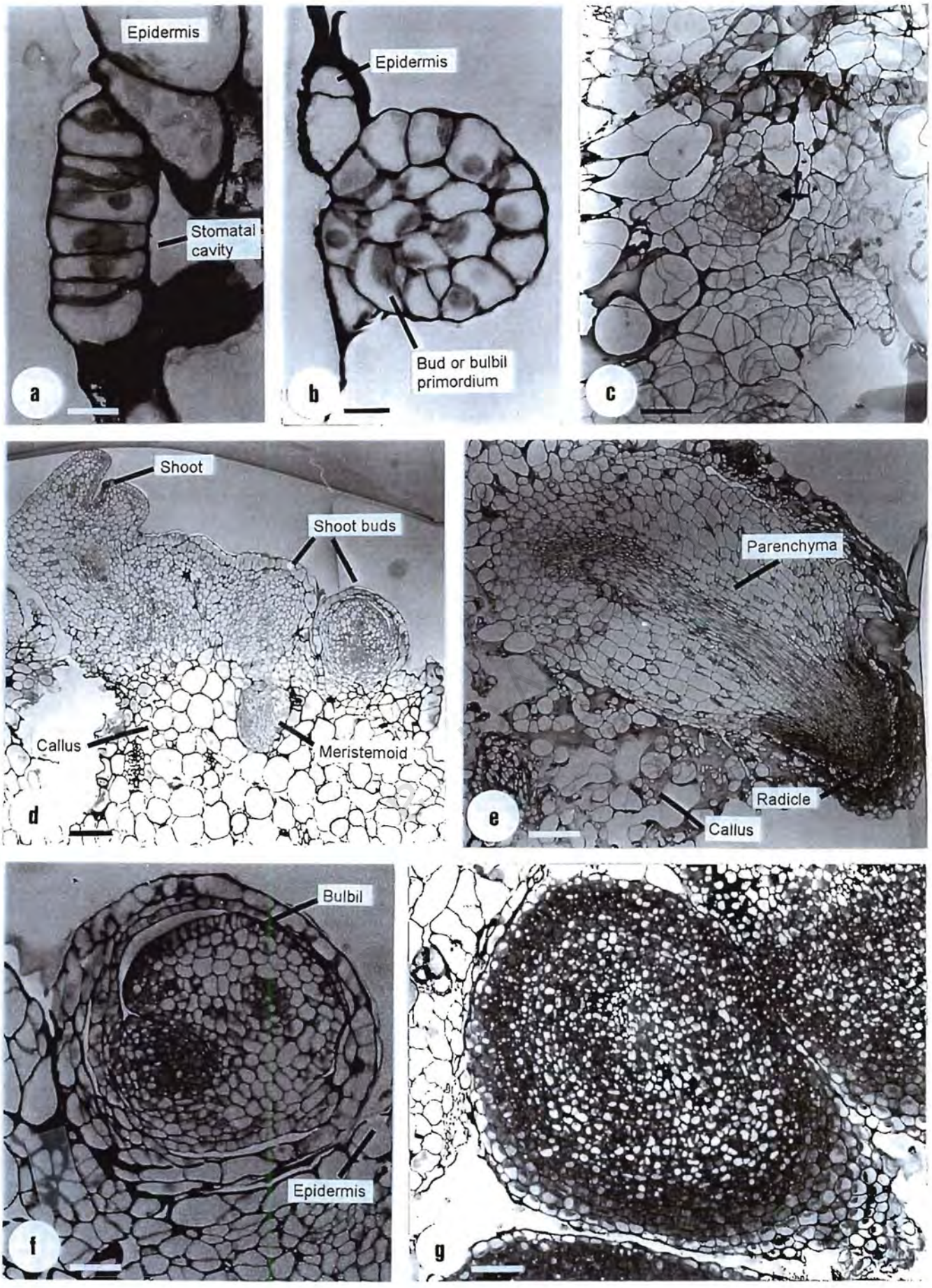

Epidermis
Stomatal cavity
a
Epidermis
Bud or bulbil primordium
b
c
Shoot
Shoot buds
Callus
Meristemoid
d
Parenchyma
Radicle
Callus
e
Bulbil
Epidermis
f
g

Figure 5

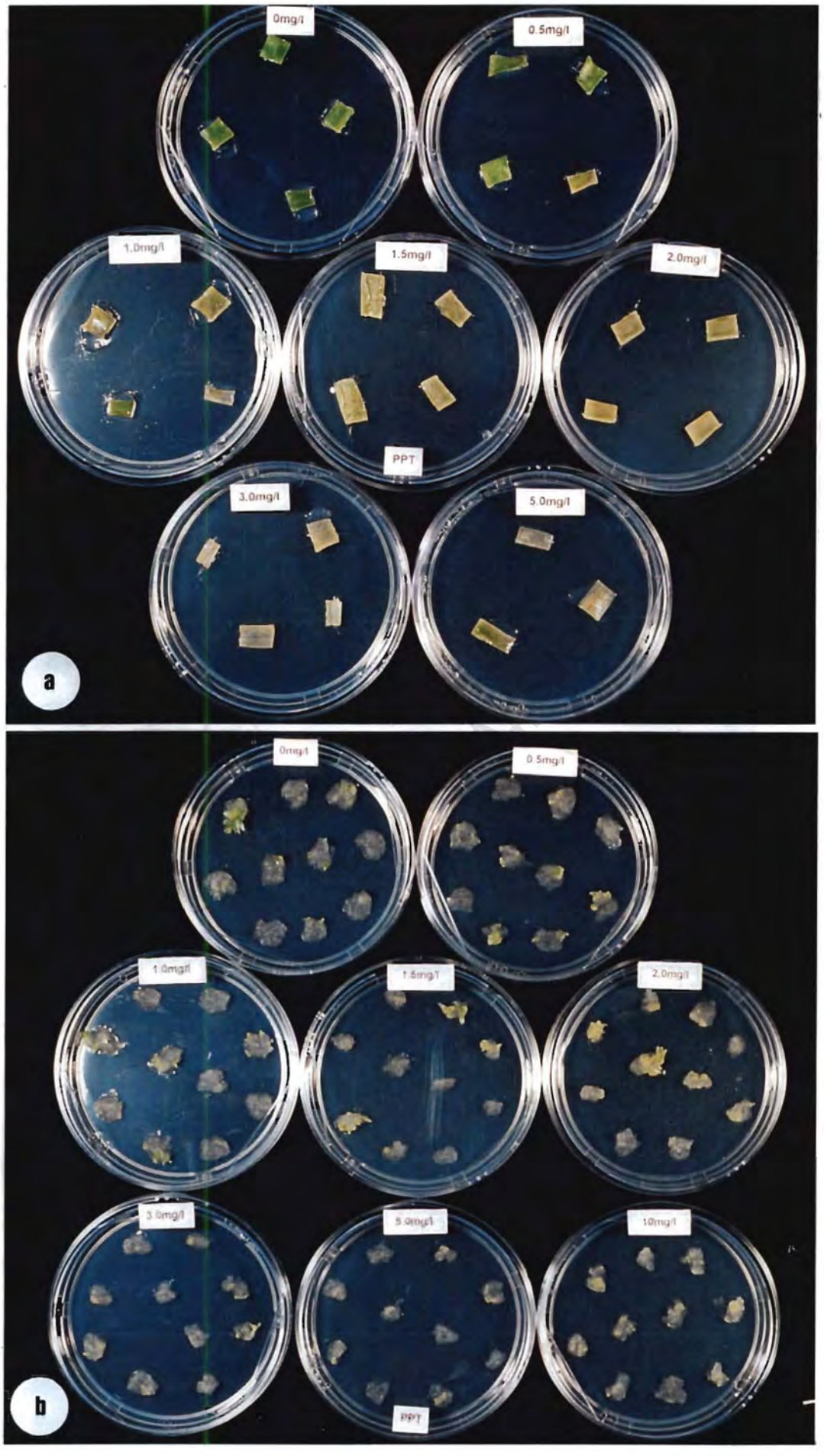

0mg/l
0.5mg/l
1.0mg/l
1.5mg/l
2.0mg/l
PPT
3.0mg/l
5.0mg/l
a
0mg/l
0.5mg/l
1.0mg/l
1.5mg/l
2.0mg/l
3.0mg/l
5.0mg/l
10mg/l
PPT
b

Figure 6

Regeneration occurred in friable callus (Figure 4d left panel), after the initiation of cell division and the formation of meristemoids and was confirmed anatomically (Figure 5c). Figures 4d (right panel) and 4e (left panel) are representative of later stages where meristemoids have developed into adventitious buds from which shoots (Figures 4f, 5d) may develop. These zones or clusters of cells with enhanced meristematic activity also served as the primordia of somatic embryos. Often, these meristemoids were distinguished by the presence of chlorophyll in their cells.

3.4.5 Optimisation of a selection protocol

Initially shoots regenerated from leaf pieces cultured on 2.5 µM PPT or less, but became discoloured and died as ammonium built up in the leaves (Figure 6a). Green meristematic centres and shoots differentiated within the first few weeks from the callus, but became etiolated or discoloured after more than four weeks on levels of 5.0 µM PPT or greater (Figure 6b). Concentrations in excess of 7.5 µM for leaf pieces and 15.0 µM for callus tissue were so toxic that no greening or regeneration of shoots was observed.

3.5 Discussion

3.5.1 Regeneration

As stated previously, leaf explants of *O. thyrsoides* respond well to culture *in vitro* (Hussey, 1976; Klesser and Nel, 1976; Nel, 1981). In this study, the protocols of Hussey (1976) and Nel (1981) were compared by testing the omission or presence of different concentrations of BA and NAA in the initiation medium.

MS supplemented with NAA (0.5 µM) and BA (2 or 9 µM), on which many rooted plants differentiated, were most efficient (Table 1). Fewer plants, which took longer to develop roots, could be regenerated on media lacking NAA. On medium containing only NAA, root formation was predominant and very few shoots were obtained (Figure 2a). This medium was avoided because in most monocotyledons, shoots seldom develop from roots. The vigour of the regenerated plants was more important than numbers, therefore medium containing 0.5 µM NAA and 2 µM BA was preferred for propagation of *in vitro* plants. Medium containing 0.5 µM NAA and 9 µM BA (Nel, 1981) was used for regeneration from greenhouse leaves.

It was possible to propagate *O. thyrsoides* on all media tested in this study, but efficiency, vigour and quantity of plants obtained differed and the end use of propagated plants should be considered in the choice of a regeneration medium.

3.5.2 Callus proliferation

Callus tissue was previously initiated from leaf pieces of *O. thyrsoides* hybrid A4 at ARC-Roodeplaat (Slabbert, pers. comm.) on callus initiation medium containing 2,4-D (9 µM) and casein hydrolysate (0.1% (w/v)). In the current study, casein hydrolysate was omitted from the callus induction medium after preliminary experiments showed that there was no difference between the amount and type of callus obtained in the absence of caseine (unpublished results). In addition, 2,4-D was compared to dicamba, NAA or IAA since less chromosomal variation has been observed with the latter three plant growth regulators as compared to 2,4-D (Hussey, 1976; Nayak and Sen, 1991; K.Kamo, pers. comm.). For the current study, the best

results were obtained with 2,4-D and dicamba compared to smaller amounts with NAA and little or no callus proliferation on IAA. Since 2,4-D is reported to induce changes in ploidy (Hussey, 1976), only callus derived from dicamba and NAA was used in regeneration studies.

Callus initiated on 2,4-D, dicamba and NAA all appeared similar. However, the roots and shoots that developed on IAA- and NAA-containing media indicated premature differentiation of callus cells, which had to be avoided for subsequent transformation and regeneration experiments. Therefore, medium containing dicamba was preferred for callus induction.

Nayak and Sen (1995) reported callus initiation from bulb scales of *O. umbellatum* on MS medium supplemented with NAA (45 µM) that could be maintained for up to five years with reduced NAA (1 µM). In the current study, NAA (27 µM) caused root formation and long culture periods were avoided to prevent changes in ploidy level from diploid to tetraploid, which leads to a reduction in regeneration potential (Hussey, 1976; Nayak and Sen, 1991). For this reason as well as the observation that the amount of the type of callus selected for transformation and regeneration in the current study deteriorated after eight weeks in culture, callus tissue were initiated frequently and used within six to eight weeks in culture.

3.5.3 Regeneration from callus

Callus derived from NAA- and dicamba-containing medium both preferred 2 µM kinetin but reacted differently to the concentration of BA in the regeneration media tested. Callus from NAA regenerated most efficiently on 2 µM BA compared to 4.5 µM BA for callus from dicamba. However, plants that regenerated from callus derived from dicamba displayed a more favourable shoot to root ratio than those did from NAA-containing medium (Figure 3). The inclination of callus initiated on NAA-containing medium to form roots on regeneration medium may be due to root meristems that developed during callus initiation (Figure 2b) and indicates that callus explants initiated in the presence of NAA does not consist of predominantly undifferentiated cells as required for biolistic transformation experiments.

Other cytokinin combinations were also successful. Shoots formed on 55% of explants initiated on dicamba and cultured on 2 µM BA (Table 3) and from 60% of explants initiated on NAA and cultured on 4.5 µM BA (Table 4). However, in these cases roots formed more often and in larger numbers than shoots and fewer plants developed compared to the media discussed previously, which were selected for subsequent studies involving regeneration from callus.

3.5.4 Histology of regeneration

It was shown that regeneration of differentiated structures from tissues of *Ornithogalum*, whether directly (Figure 1, pathway A) or indirectly (pathway B) originates from nodules of meristematic tissue. In the callus pathway, these nodules or meristemoids have presumably originated from single cells or clusters of cells which, under the influence of the chemical and physical environments, are induced to become meristematic. In pathway B, the cells of the stomatal complexes are primordially meristematic. Somatic embryos (pathway C) were a frequent feature of long term callus culture. They were also observed in tissues derived directly from the leaf meristemoids (pathway D), but the preliminary nature of this observation is emphasised by a dashed line. Pathway E, at present hypothetical but known to function in for example Larix (Klimaszewska, 1989), indicates a method by which plants could be produced from embryogenic cells via protoplasts.

Callus is a parenchyma tissue that results from the proliferation of cells at or below the surface of a wound. Monocotyledons are less responsive to wounding than dicotyledons. However, in the Liliales, wound healing includes both a closing layer and a wound periderm (Swamy and Sivaramakrishna, 1972). Figure 4a shows that this is also the case for *Ornithogalum* cultured on callus initiation medium. Callus is produced from cells proliferating near the wound surface from which friable callus (Figure 4d, left panel) was obtained. Active cell division and the formation of meristemoids was shown in this callus (Figure 5c), although without access to large numbers of serial sections the target cells it was difficult if not impossible to identify if the origin was unicellular or multicellular. These zones or clusters of cells with enhanced meristematic activity served as the primordia of plants (Figures 4f, 5d, 5f) and somatic embryos (Figures 4e, 5e). Meristemoids that originate from single cells are ideal targets for transformation studies when a single transformed callus cell can

be induced to develop into a transgenic plant. If however a meristemoid has a multicellular origin, a resulting transgenic plant will be chimeric.

3.5.5 Optimisation of a selection protocol

Initially, kanamycin was investigated as a possible selection agent but many callus and leaf explants were able to survive and regenerate into plants on levels of 0.8 mM (results not shown). This was also found to be the case for most grass species (Hauptmann *et al.*, 1988). As effective concentrations for *Ornithogalum* were excessive (1.6 mM), the effect of PPT was investigated instead.

Leaf explants and the shoots that regenerated from it became discoloured and died within 8 weeks on 10 µM PPT or more. Initially, callus explants seemed unaffected by PPT. However, green meristematic centres and shoots that regenerated within the first four weeks, discoloured on PPT concentrations in excess of 15 µM. For subsequent regeneration from transformed tissues, 10 µM PPT was used for leaf explants and 15- to 20 µM for callus.

The tissue culture system presented here for *O. thyrsoides* A2 is considered suitable for transformation, as efficient and repeatable induction of undifferentiated callus from leaf explants is possible. Plants were regenerated from adventitious shoots induced in the callus, which proved to be a suitable target tissue for transformation.

Chapter 4

Optimisation of bombardment conditions for transformation

4.1 Abstract

Transient transformation conditions were optimised for *O. thyrsoides* hybrid A2 with the GUS gene using a Biorad PDS 1000/He device. Helium pressures of 5500 to 6200 kPa, gold microprojectiles and 0.25 M osmoticum were optimal and the CaMV35S promoter was efficient in *Ornithogalum*. This was followed by bombardments for stable transformation using the *pat* herbicide resistance gene (de Block *et al.*, 1987) in two separate studies. The latter also included the Taxi method (Chen *et al.*, 1998) which entailed complexing single stranded DNA containing the *pat* gene with histone H1 prior to bombardment.

In the first study at the USDA (Beltsville, MD, USA), 346 shoots regenerated on selection medium and 137 formed roots. In the second study at the UCT (Cape Town, South Africa), 129 shoots were obtained with conventional biolistics of which 47 formed roots. With the Taxi method, 182 shoots regenerated and 74 formed roots.

4.2 Introduction

Methods for gene transfer into plant cells, particularly *Agrobacterium* and particle bombardment, are now sufficiently developed to allow transformation of essentially any plant species in which regenerable cells can be identified (Birch, 1997). *A. tumefaciens* transfers desirable genes mostly to dicotyledonous crops. Although success was obtained with a few monocots, mostly cereals, transformation is rarely routine (Gelvin, 1998). In contrast, direct gene transfer can be applied to any plant species and particle gun transformation (Sanford *et al.*, 1987; Klein *et al.*, 1987) is generally effective regardless of tissue- or cell type (Kartha *et al.*, 1989; Twell *et al.*, 1989; Vasil and Vasil, 1996).

Recently, a number of scientists have attempted to combine the best attributes of *Agrobacterium*-mediated transformation, which are high efficiency, low copy number and intact transgenes, with that of particle bombardment, which is species-independent transformation (Gelvin, 1998). Two approaches have been reported that rely on the known mechanism of T-DNA processing in *Agrobacterium* and more specifically, the nuclear targeting and uptake of T-DNA facilitated by VirD2 and VirE2 proteins. One is termed Agrolistic transformation and the other Taxi. With the former method (Hansen and Chilton, 1996; Hansen *et al.*, 1997) three separate plasmids were introduced into tobacco and maize protoplasts, two containing genes encoding VirD1 and VirD2, each under control of the CaMV35S promoter, and one containing an *nptII* gene within T-DNA borders. It was shown that in many instances the selectable marker gene integrated into the plant genomic DNA using the T-DNA borders.

In a similar attempt to overcome the disadvantages of particle gun transformation with plasmid DNA, Chen *et al.* (1998) developed the Taxi method that imitated *Agrobacterium*-mediated transformation using a macromolecular artificial T-DNA complex, as discussed in Chapter 2. They transformed *Digitaria sanguinalis* using biolistic transformation with the Taxi method and demonstrated that it can improve direct gene transfer to cereals, bypassing complications posed by the host range specificity of *Agrobacterium*.

To develop a particle bombardment protocol for a crop that has not been transformed previously, the bombardment conditions for transient expression of a reporter gene such as GUS (Jefferson *et al.*, 1987), GFP (Hasselhoff and Amos, 1995) or luciferase (Ow *et al.*, 1986) is optimised in tissues with a high regeneration potential (Sanford *et al.*, 1991). Expression from these genes are often detectable only for a few days and do not indicate the frequency of chromosomal integration. But it does confirm successful penetration of the cell wall with concomitant cell survival. Although the conditions optimal for transient expression of a reporter gene is not necessarily optimal for stable transformation (Benidiktsson *et al.*, 1995; Christou, 1995; Nandadeva *et al.*, 1999), it is the best starting point when transformation of a crop is attempted for the first time.

Ornithogalum, a monocot, is not susceptible to *Agrobacterium* infection and this chapter investigated the feasibility of biolistic transformation for *O. thyrsoides* hybrid A2. A commercial particle gun was not available at the ARC-Roodeplaat. Instead, two different PDS 1000/He biolistic® guns were used in two independent studies. One was in 1995 at the Floral and Nursery Plants Research Unit of the USDA and the other in 1996 at the Microbiology Department of the UCT. In one experiment at the UCT, the Taxi system was compared to conventional biolistic transformation. The aim was to transform A2 with the *pat* gene and to determine if the Taxi system had an advantage above conventional biolistics. All post-bombardment studies including selection, regeneration and analysis of bombarded tissues were performed at the ARC-Roodeplaat.

Bombardment conditions were optimised for several parameters by studying transient expression frequencies obtained in callus. Helium pressures were evaluated as well as the number of bombardments and type of microprojectiles that were most effective, the influence of osmoticum on transient expression frequency and finally, the efficiency of four different promoters. The best conditions were used to bombard callus tissues for stable transformation with the *pat* gene, using both conventional biolistics and the Taxi method, followed by selection of transformed tissues and regeneration of transgenic plants on medium containing PPT.

4.3 Materials and Methods

4.3.1 Preparation of callus for bombardment

For each bombardment, undifferentiated callus tissue was spread over a 30 mm diameter circle in the centre of a petri dish containing either callus induction medium or osmotic medium, which consisted of callus induction medium supplemented with equal molarities of mannitol and sorbitol combined to a final concentration of either 0.25, 0.5, 0.75 or 1.0 M. Tissues bombarded on osmotic medium were cultured on this medium for 4 h prior to and 16 h after bombardment followed either by staining or transfer to callus induction medium. During the 4 h plasmolysis pre-bombardment period, target tissues were left open in a laminar flow hood for 30 min in experiments done at the USDA or for 15 min in experiments done at the UCT, to evaporate the film of moisture that formed on the callus.

4.3.2 Bombardment protocol

Staining: Transient transformation events were visualised by staining for GUS activity, 24 h after bombardment with X-Gluc (1mM) in NaH_2PO_4 (50 mM, pH 7), Na_2EDTA (1 mM) and Sarcocyl (0.5% (w/v)), based on the report of Jefferson *et al.* (1987). Enzyme activity was expressed as the number of blue spots counted under a binocular dissecting microscope, after at least 12 h incubation at 37 °C.

Bombardment: A PDS 1000/He Biolistic gun® (Biorad, Richmond, CA, USA) was used for transformation and tissues were bombarded with 1 µm diameter gold or tungsten microprojectiles (Biorad) with a travelling distance of 9 cm. Each treatment contained 5 to 10 replicates.

DNA constructs: All plasmid DNA used for bombardments were isolated by alkaline lysis followed by cesium chloride gradient purification (Sambrook *et al.*, 1989). Both plasmids pBI221 (Clontech, Palo Alto, CA, USA) and pDM327 (gift from David McElroy), used to optimise bombardment conditions, contained the CaMV35S promoter upstream of either the GUS reporter gene (Jefferson *et al.*, 1987) or a *bar*GUS fusion. For promoter studies, pBI505 (Russel *et al.*, 1992), pACT1D (McElroy *et al.*, 1991) and pubi7 (Garbarino *et al.*, 1995) were used in addition to

pBI221 and pDM327. These additional constructs all contained the GUS gene under control of the eCaMV35S, rice act and potato ubi promoters, respectively. p35SAC (Agrevo, Frankfurt, Germany), containing the *pat* selectable marker gene (de Block *et al.*, 1987) under control of the CaMV35S promoter was used for stable transformation. All the constructs used for bombardment are illustrated in Figure 7.

For optimisation, plasmid DNA was precipitated onto tungsten or gold particles according to the method of Klein *et al.* (1987). Ten µg of plasmid DNA was precipitated with 50 µl $CaCl_2$ (2.5 M) and 20 µl spermidine (100 mM) onto 3 mg of sterile washed particles. After brief centrifugation, the supernatant was discarded and DNA-coated particles resuspended in 60 µl absolute EtOH and 10 µl aliquots were dried on macrocarriers (Biorad) for bombardment. Various helium pressures (2800, 5500, 6200 and 9000 kPa), number of bombardments (single or double), type of microprojectiles (tungsten or gold), effect of osmoticum (0, 0.25, 0.5, 0.75 or 1.0 M) and the efficiency of different promoters - CaMV35S, eCaMV35S, rice act and potato ubi - were evaluated.

For stable transformation, DNA was precipitated using either the method described above or the Taxi protocol (Chen *et al*, 1998). For the latter method, p35SAC DNA was linearised with Hind III, diluted (6 µg in 75 µl 0.1 x TE), denatured by boiling for 10 min, mixed with purified sea-urchin histone H1 (von Holt *et al.*, 1989; 18 µg in 25 µl 0.1 x TE) and incubated on ice for 30 min. The DNA-histone H1 complex was added to sterile, washed gold particles (3 mg) and mixed at room temperature for 45 min. After brief centrifugation, the supernatant was discarded and DNA coated gold particles resuspended in 60 µl sterile H_2O. Ten µl aliquots of the suspension were placed on macrocarriers and allowed to dry before bombardment.

Culture of bombarded tissues: For regeneration of transformed plants, bombarded callus tissues were transferred to selection medium consisting of MS medium with 4.5 µM BA, 2 µM kinetin and PPT (10 µM for tissues bombarded at the USDA; 15 µM for tissues bombarded at the UCT), seven days after bombardment. Shoots were transferred to rooting medium (MS medium with 12.5 µM PPT for plants from the USDA or 20 µM PPT for plants from the UCT) after at least eight weeks on selection medium.

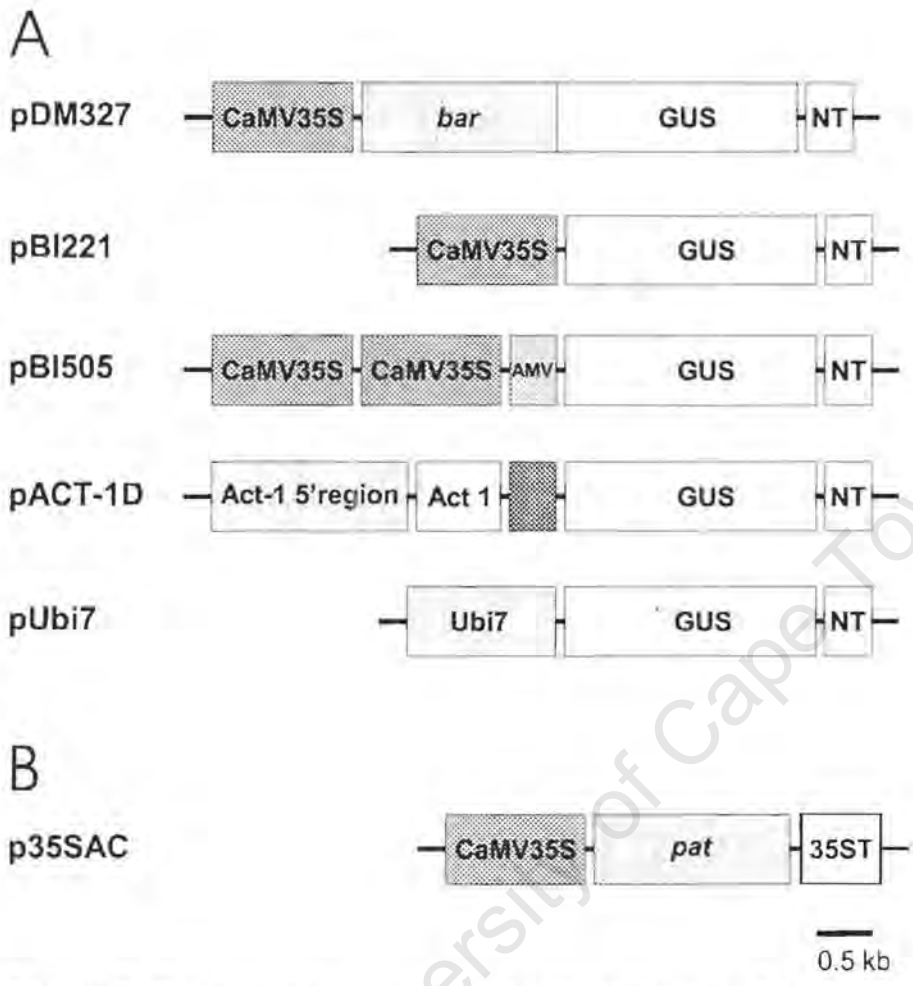


Figure 7 Gene constructs used for biolistic transformation of *O. thyrsoides* A2. Vector backbones each contained an ampicillin resistance selectable marker gene and the *colE* plasmid origin of replication.

(A) Optimisation of transient transformation
pDM327: CaMV35S promoter – *bar*GUS fusion – NT
pBI221: CaMV35S promoter – GUS – NT
pBI505: eCaMV35S promoter - AMV leader – GUS – NT
pACT1D: act 5' region - act promoter - acti1st intron - GUS – NT
pubi7: potato ubi promoter – GUS – NT

(B) Stable transformation
p35SAC: CaMV35S promoter – *pat* – CaMV35S terminator

Callus tissue bombarded at the USDA that became contaminated with endogenous bacteria when cultured on selection medium, were often in danger of being overgrown. To save the cultures, selection pressure was withdrawn for three weeks and 50 µM cefotaxime was included in the culture medium. Tissues were subcultured every five days for three weeks onto fresh antibiotic-containing medium to eliminate bacterial growth. These cultures were submitted to a second round of selection for four months on 12.5 µM PPT.

4.4 Results

4.4.1 Optimisation of transient expression

The first set of experiments was conducted at the USDA with six- to eight-week old callus. Transient transformation frequencies obtained from optimisation experiments for five different parameters are presented in Table 5 and were calculated as the mean number of blue spots observed for five- to ten bombardments after staining with X-Gluc as illustratd in Figure 8. Non-bombarded tissue did not form blue spots or stained blue otherwise (results not shown).

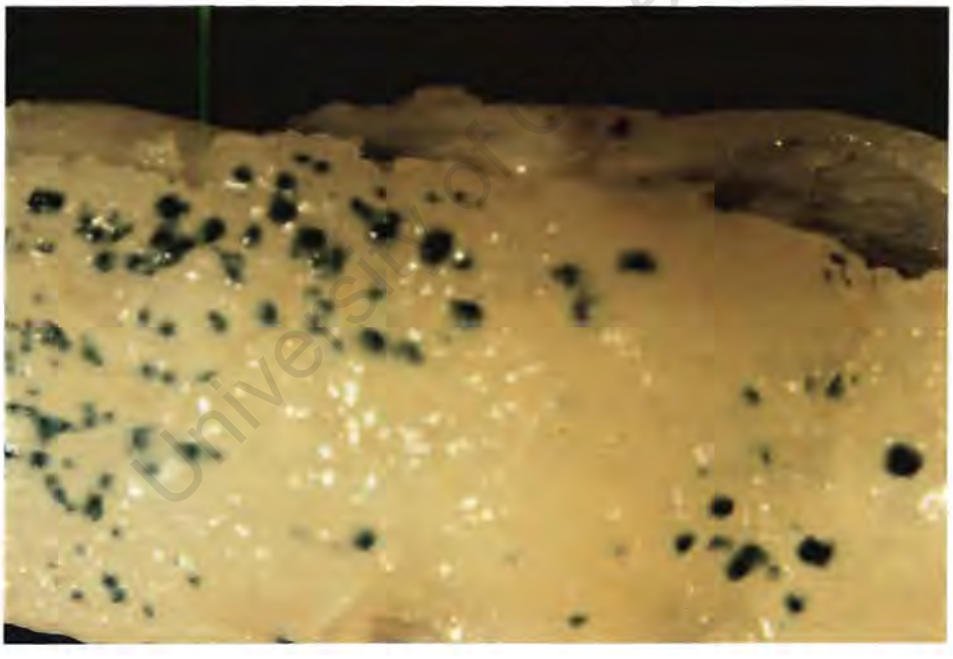


Figure 8 Transient transformation of callus tissue of *O. thyrsoides* A2. Each blue spot represents a transient transformation event, visualised by GUS staining with X-Gluc 24 h after bombardment.

Helium pressure was optimised first and both 5500 and 6200 kPa yielded many transformation events (Table 5, experiment 1). Single and double bombardments, where the same target tissue was bombarded twice, were compared next. Single bombardments at 5500 kPa (140 spots) were more efficient than either single (76 spots) or double (92 spots) bombardments at 2800 kPa (experiment 2). Therefore, pressure was set at 5500 kPa in subsequent experiments. In experiment 3, tungsten and gold microprojectiles (Biorad) gave comparable results (67 and 88 spots, respectively), but Aldrich gold (Aldrich Chemical Co, Milwaukee, WI, USA) adhered to the sides of reaction tubes and gave very low transformation frequencies (7 spots). Callus induction medium supplemented with 0.25 M osmoticum, produced substantially more blue spots than 0, 0.5, 0.75 or 1.0 M (experiment 4).

Finally, promoter studies showed that the eCaMV35S promoter was approximately twice as efficient as the standard CaMV35S, rice act- and potato ubi promoters, which all gave comparable results (experiment 5). Of the latter three promoters, CaMV35S consistently resulted in the highest maximum number of transformation events. This was confirmed by results from bombardments where pDM327, which contained a *bar*GUS fusion driven by CaMV35S, were compared to pACT1D and pubi7.

Results of transient transformation studies performed in the second study at the UCT are presented in Table 6. Single (98 spots) and double bombardments (95 spots) at 6200 kPa gave similar values, which were better than both single (84 spots) and double bombardments (69 spots) at 9000 kPa, confirming that 6200 kPa were better and that double bombardments did not improve transformation frequencies. Gold microprojectiles performed better than tungsten. No osmoticum, in combination with 30 min drying in the laminar flow hood during the pre-bombardment plasmolysis period, yielded more spots than 0.25 M osmoticum.

Table 5 Transient GUS expression as a function of rupture disc pressure, osmoticum, promoter and plasmid bombardment parameters. Values indicate the numbers of GUS-positive foci and are the means ± SD of 5 to 10 replicates.

Experiment	Bombardment conditions				Transient GUS expression
	Pressure kPa	Osmoticum M	Promoter	Plasmid	
1	2800	0	CaMV35S	pDM327	76 ± 45
	2800	0	CaMV35S	pBI221	5 ± 2
	5500	0	CaMV35S	pDM327	140 ± 37
	5500	0	CaMV35S	pBI221	87 ± 19
	6200	0	CaMV35S	pDM327	177 ± 88
	6200	0	CaMV35S	pBI221	39 ± 30
2	2800[a]	0	CaMV35S	pDM327	92 ± 80
	5500[a]	0	CaMV35S	pDM327	66 ± 15
3	2800[b]	0	CaMV35S	pDM327	67 ± 80
	5500[b]	0	CaMV35S	pDM327	88 ± 77
4	5500	0	CaMV35S	pDM327	10 ± 13
	5500	0	CaMV35S	pBI221	3 ± 3
	5500	0.25	CaMV35S	pDM327	59 ± 30
	5500	0.25	CaMV35S	pBI221	59 ± 24
	5500	0.5	CaMV35S	pDM327	1 ± 2
	5500	0.5	CaMV35S	pBI221	7 ± 7
	5500	0.75	CaMV35S	pDM327	0
	5500	0.75	CaMV35S	pBI221	1 ± 1
	5500	1.0	CaMV35S	pDM327	1 ± 1
	5500	1.0	CaMV35S	pBI221	0
5	5500	0	CaMV35S	pDM327	89 ± 34
	5500	0	CaMV35S	pBI221	86 ± 34
	5500	0	eCaMV35S	pBI505	217 ± 34
	5500	0	Rice act	pACT1D	74 ± 39
	5500	0	Potato ubi	pubi7	72 ± 28

[a] double bombardments, otherwise single
[b] tungsten microprojectiles, otherwise gold

Table 6 Optimisation of transient transformation frequencies[a] for *O. thyrsoides* A2 performed at the UCT using pBI221. Values indicate the numbers of GUS-positive foci and are the means ± SD of 5 to 10 replicates.

Bombardment conditions		Transient GUS expression
Pressure kPa	Osmoticum M	
6200	0	98 ± 46
9000	0	84 ± 38
6200	0[a]	95 ± 42
9000	0[a]	69 ± 31
6200	0[b]	11 ± 10
6200	0	25 ± 24
6200	0	50 ± 36
6200	0.25	29 ± 30
6200	0.5	19 ± 22
6200	0.75	13 ±10
6200	1.0	6 ± 6

[a] double bombardments, otherwise single
[b] tungsten microprojectiles, otherwise gold

4.4.2 Bombardment for stable transformation

Bombardment conditions used for stable transformation were 5500 kPa at the USDA and 6200 kPa at the UCT, gold microprojectiles, CaMV35S promoter, single bombardments and 0.25 M osmoticum with 15 (UCT) or 30 min (USDA) drying in the laminar flow hood during the pre-bombardment plasmolysis period. At the UCT, additional variables were tested in one experiment, i.e. using 7600 instead of 6200 kPa and transformation with the Taxi. The numbers of bombardments performed at each location are presented in Tables 7, 8 and 9.

At the USDA, a total of 92 plates were bombarded on six different dates (Table 7). Thirty-five plates (36%) had to be discarded three weeks after bombardment due to contamination and 57 plates remained for regeneration of plants from transformed tissues. At the UCT, 72 plates were bombarded with conventional biolistics of which 44 (61%) contaminated leaving 28 plates for regeneration studies on selection medium (Table 8). For the Taxi, 123 plates were bombarded, of which 54 (44%) became contaminated, leaving 69 plates for regeneration studies (Table 9).

4.4.3 Regeneration of PPT-resistant plants from bombarded tissues

The tissues bombarded at the USDA and cultured on regeneration medium supplemented with 10 µM PPT were being overgrown by an endogenous bacterial contaminant. Although 36% of the bombarded callus had to be discarded (Table 7) the remaining tissues were retained by culture in the presence of antibiotic without selection pressure for three weeks. During the second round of selection on 12.5 µM PPT for four months, a total of 346 shoots regenerated from the 57 plates retained after bombarded. Of these, 135 developed roots on medium supplemented with 12.5 µM PPT (Figure 9a).

Table 7 Bombardment of callus tissue for stable transformation at the USDA.

Date	Bombardments	Bombarded cultures[a] retained
7.12.95	12	11
8.12.95	12	0
12.12.95	24	11
13.12.95	12	11
14.12.95	20	16
16.12.95	12	8
Total	92	57

[a]Bombarded cultures that were contaminated within three weeks after bombardment, were discarded

Table 8 Bombardment of callus tissue for stable transformation at the UCT.

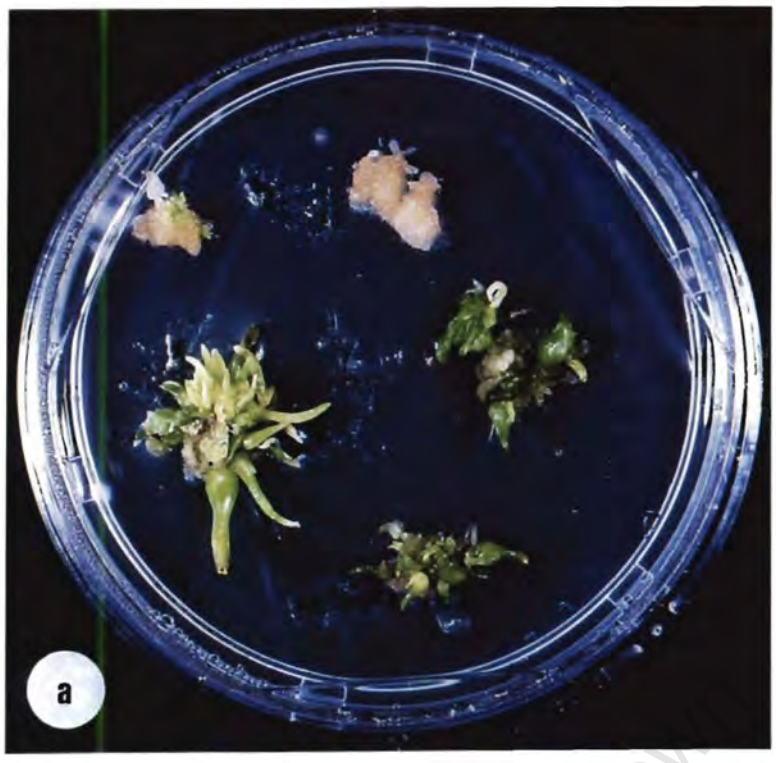
Date	Pressure kPa	Bombardments	Bombarded cultures[a] retained
12.12.96	6200	12	1
16.12.96	6200	12	8
17.12.96	6200	24	9
18.12.96	7600	24	10
Total		72	28

[a]Bombarded cultures that were contaminated within three weeks after bombardment, were discarded

Table 9 Bombardment of callus tissue for stable transformation at the UCT using the Taxi protocol.

Date	Pressure kPa	Bombardments	Bombarded cultures[a] retained
12.12.96	6200	33	21
16.12.96	6200	18	9
17.12.96	6200	36	23
18.12.96	7600	36	16
Total		123	69

[a]Bombarded cultures that were contaminated within three weeks after bombardment, were discarded



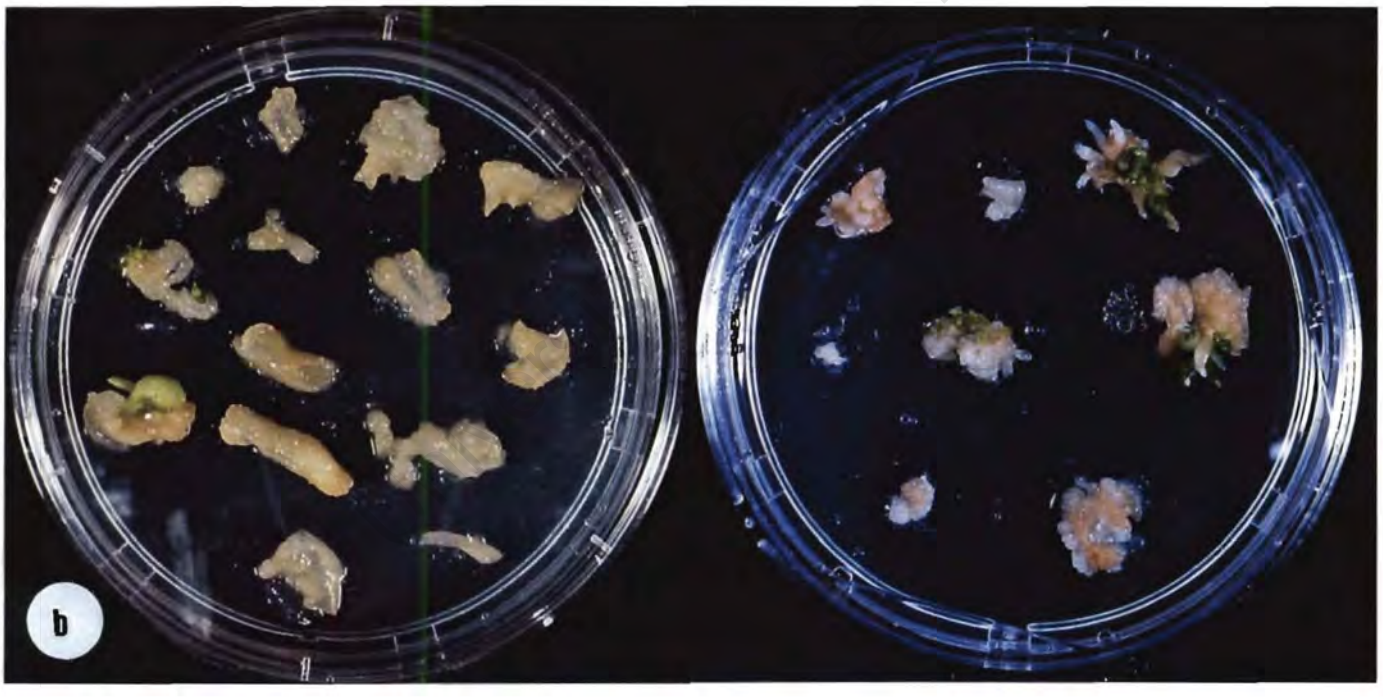


Figure 9 Examples of the frequency of regeneration of plants obtained from callus bombarded at:

(a) the USDA and cultured for three weeks on regeneration medium with 10 µM PPT, three weeks without PPT and another four months in the presence of 12.5 µM PPT

(b) the UCT and cultured for three months on 15 µM PPT

In experiments performed at the UCT only 28 plates of tissue (39 %) of the 72 that were bombarded with conventional biolistics, remained free of bacteria (Table 8). Stricter selection was applied to these tissues and after three months on medium containing 15 µM PPT, a total of 72 shoots had developed, which increased to 129 in the following month (Figure 9b). Of these shoots, 74 developed roots within four months in culture.

Sixty-nine plates of callus tissue (56 %) were retained after bombarded with the Taxi method at UCT. A total of 182 shoots developed within three months from the callus cultured on selection medium containing 15 µM PPT. After a further two months, 142 of these shoots had formed roots, but with increased selection pressure of 20 µM PPT, only 33 survived.

4.5 Discussion

Prior to the two studies described in this thesis, a particle inflow gun (PIG) was constructed at ARC-Roodeplaat according to the specifications of Brown *et al.* (1994). Although this device was shown to work in our laboratory for onion bulb scales, tobacco cell suspensions, maize- and wheat immature embryos (de Villiers and Laib, 2000), it did not work for *Ornithogalum*. During the six months preceding the USDA study, 23 experiments, each consisting of 24 to 36 bombardments were conducted with the PIG on both callus and leaf explants of *O. thyrsoides* A2. Variables tested included helium pressure, microprojectile travelling distance, type of microprojectiles, inclusion of a nylon mesh to diffuse the helium shock wave, the effect of osmoticum prior to bombardment, using callus at different stages of development and comparison of the CaMV35S (pBI 221) and maize ubi (pAHC25; Christensen and Quail, 1996) promoters. To ensure that the problem was not due to the quality of the DNA used, precipitation efficiency was monitored, integrity of the DNA was verified and various DNA isolation protocols compared. In more than 70% of cases, no transient transformation were observed and for both leaf and callus explants the largest number of spots observed were nine and eight, respectively. It was concluded that either the target tissues were not amenable to gene transfer with the PIG or that the helium shock wave caused by this device damaged the target tissue to the extent that no transformed cells survived. As a result, the two studies with the PDS 1000/He devices were undertaken to determine if the callus chosen as explants in this study was amenable to transformation.

4.5.1 Optimisation of transient expression

In general, transformation frequencies vary between bombardments (Birch and Bower, 1994), as can be seen from the standard deviations presented in this study. However, if high transformation frequencies can be achieved (in excess of 50 spots per bombardment), and adequate quantities of target tissue and DNA are available, this is not a serious problem for generating transgenic plants (Birch and Bower, 1994). For this study, sufficient quantities of target callus tissue were available and this was used in the most sensible way for the optimisation of transient transformation.

In both sets of experiments discussed in this chapter, helium pressure was optimised first. At the USDA 5500 kPa was selected for subsequent experiments (Table 5). At the UCT, 5500 kPa was not tested because the rupture disks were not commercially available. In this study, 6200 kPa and 9000 kPa was tested instead; 6200 kPa yielded the largest number of blue spots and was used in subsequent experiments at this location (Table 6). Higher, rather than lower helium pressures were tested in the second study, as the USDA study indicated that, higher maximum numbers of transformation events were mostly obtained with increased (5500 kPa; 140 spots and 6200 kPa; 177 spots) rather than lower pressure (2800 kPa; 76 spots). For this reason and the fact that the best transient transformation conditions do not always yield the highest number of stable transformation events (Bower and Birch, 1994; Benidiktsson *et al.*, 1995; Christou, 1995; Nandadeva *et al.*, 1999), 7600 kPa was used for one set of stable transformation experiments (Table 9) to determine if higher pressure yielded more stable transformation events.

At the USDA, transient transformation studies were performed with the CaMV35S promoter (pBI221 and pDM327; Table 5). pDM327 seemed more efficient than pBI221, although both contained the CaMV35S promoter. It can be ascribed to the *bar*GUS fusion in pDM327, which stabilises the GUS enzyme in transformed cells and protects it against cellular proteases. This allows prolonged GUS activity, which leads to increased substrate turnover, resulting in more pronounced blue spots (K. Kamo and D McElroy, pers. comm.). In p35SAC, which contains the *pat* gene and was used for stable transformation, as well as in pBI221, the CaMV35S promoter does not drive a gene encoding a fusion protein. Therefore pBI221 was used in the final set of optimisation studies at both the USDA (Table 5) and at the UCT (Table 6) as it was considered to give a better reflection of expression of transgenes in the target tissue.

Promoter studies showed that the eCaMV35S promoter was about twice as efficient as the standard CaMV35S, rice-act and potato ubi promoters, which all gave comparable results (Table 5, experiment 5). As CaMV35S (in pBI221 and pDM327) consistently yielded the highest maximum number of transformation events, it appears that with respect to promoter preference, *Ornithogalum* reacts more like a

dicotyledon than a graminaceous monocotyledon. This is supported by the reports of Wilmink *et al.* (1995), who found similar trends in various species of the Liliaceae, and Kamo *et al.* (1995) who showed that CaMV35S acted efficiently in *Gladiolus*.

The aim of the study at the UCT was to verify the transient transformation results obtained at the USDA and to compare the Taxi to conventional biolistics in stable transformation studies. Only pBI221 was used for optimisation studies in this case (Table 6).

Surprisingly, including osmoticum at the UCT did not correlate with the results from the USDA study where 0.25 M osmoticum made a dramatic difference to transient transformation frequencies (Table 5, experiment 4). At the UCT, the target tissues were left open for 30 min in a laminar flow hood on osmotic medium during the 4 h pre-bombardment plasmolysis period to allow excess moisture to evaporate from the target tissue. This was done to prevent the possible deceleration of microprojectiles by the thin film of moisture that formed on target tissue during plasmolysis, which could prevent entry of microprojectiles through plant cell walls (Bond, 1993). At the UCT, this "drying" of tissues seemed to be effective, as better transient transformation results (50 spots) were obtained with drying than with 0.25 M osmoticum (29 spots). However, the combined effect of drying and osmoticum was detrimental, as transformation frequencies decreased with increasing osmoticum. This was probably due to a stronger airflow in the laminar flow hood at the UCT than at the USDA, causing cell damage when combined with osmoticum. As a result, drying time was shortened in stable transformation studies at the UCT to 15 instead of 30 min.

It can be said that the results obtained at the UCT followed the same trends as those obtained at the USDA except for the effect of osmoticum. Promoter studies showed that eCaMV35S was more efficient than standard CaMV35S but, as the *pat* gene construct (p35SAC) was available only under control of normal CaMV35S, this was the only promoter applied for stable transformation. It is recommended that eCaMV35S should be the preferred promoter in future transformation studies with *Ornithogalum*.

4.5.2 Bombardment for stable transformation

In both studies, a large proportion of the bombarded tissues were lost after a few weeks of culture on selection medium because of bacterial contamination. The contaminant, identified as a *Bacillus* sp. (N Mienie, ARC-Roodeplaat, pers. comm.), occurs often in tissue culture of *Ornithogalum* but does not prevent efficient micropropagation (D Wilson, Hadeco; pers. comm.). However, all the steps involved in transformation stress explants and target tissues severely through callus induction, plasmolysis, bombardment, selection pressure and regeneration. It is postulated that the combined effect of these procedures caused latent bacterial contamination to manifest as severe bacterial infections. This problem was worse in other *Ornithogalum* lines tested for callus induction prior to this study (results not shown) and the importance of pathogen-free starting material cannot be over-emphasised.

The severe bacterial infection in the tissues bombarded at the USDA and analysed at the ARC-Roodeplaat can be explained by the additional stress applied to these tissues during air transport from the USA to South Africa, which could have caused re-emergence of the bacteria.

For stable transformation with the Taxi, linearised plasmid DNA and purified histone H1 were mixed to allow protein-DNA binding to occur. This resulted in a soluble ssDNA-histone H1 complex. Chen (1996) established that the optimal mass ratio of histone H1 (Mw = 2.1×10^4) to ssDNA (5.1 kb, Mw = 1.78×10^6) is 2.5:1; representing a molar ratio of approximately 200:1. At this binding ratio, one histone molecule binds to about 25 bases of the ssDNA, forming a soluble complex. Chen postulated that histone H1, through interaction of either of its basic N- or C-terminal arms or both covered the whole ssDNA molecule and protect it against nuclease attack. In this study, p35SAC (3. 99kb) was used at a ratio of approximately 3:1.

It would have been valuable if herbicide resistant transgenic plants that also expressed GUS were included in the current study to simplify verification of the transgenic nature of regenerated plants. This need for GUS-containing transgenic plants became more pronounced during molecular and transgene expression analyses (Chapter 5). An attempt was made to obtain such plants in 1997, using a

PDS 1000/He gun at AECI (Modderfontein, South Africa) but no plants regenerated from 80 plates co-bombarded with p35SAC and pBI505, the latter containing GUS under control of the eCaMV35S promoter. In this particular study, transient transformation frequencies were also very low. It is postulated that the vacuum pump attached to this particular particle gun was not powerful enough to generate a vacuum of 85 kPa within a short enough period of time. The extended time needed to obtain a proper vacuum probably caused excessive tissue damage with concomitant reduced survival and regeneration potential.

4.5.3 Regeneration of PPT-resistant plants from bombarded tissues

As discussed previously, regeneration from bombarded callus can occur from three possible events. First, no transformation or transient transformation may occur, giving rise to escapes that usually do not survive selection. Second, transformation of a single cell within a meristemoidal centre can give rise to a chimeric plant, containing both transformed and non-transformed cells. The number of transformed cells may be enough to enable the plant to survive selection pressure. These plants are difficult to distinguish from completely transformed plants that represent a third possibility, namely transformation of a single cell and its subsequent division into a meristemoid and eventually a transgenic plant.

Studies with herbicide showed that 5 µM PPT or more was lethal to callus (Figure 6b) but to prevent regeneration of chimeras on selection medium, 10 or 12.5 µM PPT was included in the regeneration medium for tissues from the USDA. However, many plants (137) regenerated on this selection medium within seven months, including the three months without selection pressure. It is possible that transformed, herbicide resistant callus cells acted as a "filter" and protected regenerating plants by reducing the amount of PPT that reached differentiating meristemoids. Alternatively, many of these plants might have been chimeras. Therefore, selection pressure was maintained without interruption on 15 µM PPT for callus bombarded at the UCT where only 72 shoots formed within three months. This increased to 129 in the following month of which 47 developed roots within six months in culture. For the Taxi, 182 shoots had regenerated within three months after transformation, and 74 had formed roots within six months.

The fact that so many plants regenerated and survived on 12.5 µM PPT, but not on 15 µM PPT as illustrated in Figure 9, indicated that most of the plants that regenerated from the USDA study were either chimeras or escapes. This illustrated the importance of stringent selection for *Ornithogalum*. These results correspond partly with that of Watad *et al.* (1998) who found that from 342 shoots that regenerated from bombarded callus of *Lilium*, 55 plantlets survived selection but only 19 contained the *pat* gene. Therefore the remaining 36 plants were either escapes or chimeras with a very low percentage of transformed cells, which were not detected by PCR. This possibility was avoided for *Ornithogalum* by strict adherence to the selection regime in the latter part of this study and plants that survived on selection medium for four months after transformation but were not able to grow taller than approximately 1 cm, were assumed to be chimeric. Rooted plants obtained after strict selection that grew 5 cm or taller were propagated *in vitro* using leaf explants on regeneration medium supplemented with 10 µM PPT and subsequently analysed by PCR for the presence of the *pat* gene (Chapter 5).

Results of this study indicated that conventional biolistics and the Taxi was equally efficient with regard to the regeneration of shoots and rooted plants on selection medium when the mean number of shoots that had formed after three months and the number of rooted plants that regenerated per bombarded plate after six months on selection medium was calculated for each treatment. For the conventional method, approximately 2.6 shoots formed within 3 months (72 on 28 plates) and 1.7 rooted plants (47) regenerated per bombardment, compared to 2.6 shoots (182 on 69 plates) and 1.1 (74) rooted plants per bombardment for the Taxi method.

Chapter 5

Verification of transformation

5.1 Abstract

Putative transgenic plants of *O. thyrsoides* A2, obtained from both the USDA and the UCT studies, were propagated and submitted to molecular analyses. Results of PCR analyses of plants from the USDA study were inconsistent. In contrast, one plant from the UCT study, designated T1, and five clones propagated from it, contained the *pat* gene. This single transgenic event and 17 of the 37 clones derived from it were analysed in further experiments.

Southern and northern blots of eight clones of T1 showed that the *pat* gene was integrated in the genomic DNA and confirmed that RNA was transcribed. Leaves of 13 clones painted with herbicide as well as ELISA of seven of these clones confirmed expression of the PAT enzyme in tissues of these plants. Finally, greenhouse trials showed that transgenic plants looked normal with regard to leaf and flower morphology. Leaves of these plants were reintroduced to *in vitro* culture and plants could regenerate from them on medium supplemented with 10 µM PPT.

5.2 Introduction

Phenotypic and molecular analyses of transgenic plants are necessary to determine (i) if the transgene, in this case the *pat* gene, is present, (ii) if it integrated in the genomic DNA, (iii) the number of copies that integrated and (iv) if the transgenic trait is expressed.

PCR (Mullis and Faloona, 1987) and Southern blotting (Southern, 1975) are widely used for the first step. Although PCR is the easiest method to determine if the *pat* gene is present in a plant, it does not prove that it is in every cell or that it integrated in the genomic DNA. For this reason, genomic Southern blotting remains the most reliable technique to prove stable integration of a transgene (Schaff, 1991). Proof of stable genomic insertion of a transgene is still no guarantee that the transgenic trait will be expressed correctly, as was described in Chapter 2. Northern blotting and RT-PCR can show if RNA is transcribed from the transgene and whether the transcription is constitutive, tissue specific or does not occur at all. A northern blot is more informative than RT-PCR as it provides quantitative information on both the activity of the promoter that drives transcription from the gene and the level of transcription. Western blot, ELISA or an appropriate enzyme assay can be used to confirm the presence of the protein product. The first two techniques require specific antibodies directed against the target protein to verify that it is synthesised and to quantify the amount synthesised. If the protein is an enzyme, such as PAT, an assay will confirm the integrity, correct conformation and biological activity.

Transgenic plants and their progeny also need to be analysed in greenhouse and field trials to confirm the correct phenotype and expression of the transferred trait under field conditions. For *Ornithogalum* hybrids that are propagated vegetatively from stable clones, as is the case for A2 which is sterile, this entails confirmation of normal phenotype and flower morphology. However, if the target crop is propagated through seed, additional genetic analysis is required to determine inheritance patterns and stability of the transgenic trait in the progeny.

This chapter reports on the molecular analyses using PCR, Southern and northern blots, ELISA, leaf assays and greenhouse trials of one putative transgenic plant of *O. thyrsoides* A2, designated T1, and of clones that were propagated from leaves of it.

5.3 Materials and methods

5.3.1 *In vitro* propagation of PPT-resistant plants

PPT-resistant plants that regenerated from bombarded callus cultured on selection medium were propagated *in vitro* from at least five leaf explants of each plant on regeneration medium (Chapter 3) containing 5 µM PPT for plants from the USDA- and 7.5 or 10 µM PPT for plants from the UCT studies. Leaf explants from non-transformed plants were cultured on regeneration medium with (negative control) or without PPT (regeneration positive control) to compare the effectiveness of the selection agent and the efficiency of regeneration from PPT-resistant putative transgenic leaf tissues. Explants were transferred to fresh medium every four weeks and, for rooting, to MS medium with 5 (USDA) or 10 µM PPT (UCT) after eight weeks. Once the plants had formed roots they were maintained on MS medium supplemented with 20 µM PPT.

Twenty-three clones from T1 were transferred to a greenhouse. After eight weeks, a 2- to 5 cm long piece of a leaf from each plant was surface sterilised, cut into 1 cm squares and cultured on regeneration medium with 10 µM PPT to provide support material for each clone.

5.3.2 PCR analyses for the presence of the *pat* gene

Rooted plants that regenerated from bombarded callus cultured on selection medium were grown to a height of 5 cm. DNA was isolated from 1 cm of a leaf of each plant and used for PCR analysis with primers directed against the *pat* gene.

DNA extraction. DNA was extracted according to Doyle and Doyle (1990). Individual leaf samples (50 to 150 mg) were ground to a paste in eppendorf tubes with a mortar and carborandum (180 gri). Extraction buffer (400 µl of 3% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, pH 8.0 and 0.2% (v/v) ß-mercapto-ethanol) was added and each sample was incubated at 60 °C for 30 min. An equal volume of

chloroform:isoamylalcohol (24:1) was added, mixed thoroughly and centrifuged for 5 min at 10 000 x g. The supernatant was transferred to a clean tube, the DNA was precipitated with 250 µl isopropanol, centrifuged for 10 min at 10 000 x g and washed with 500 µl 70% ethanol. DNA pellets were resuspended in 50 µl TE (10 mM Tris, pH 8.0, 1 mM EDTA) and the amount of DNA in each sample (diluted 1:1000 in 10 mM Tris, pH 7.4, 0.1 M NaCl, 10 mM EDTA) was determined with a fluorometer (Sequoia-Turner, Unipath, CA, USA).

PCR conditions. PCR was performed on 30 ng of genomic DNA in a MiniCycler® (MJ Research, San Francisco, CA, USA). Primers (synthesised by Genosys, Whitehead Scientific, South Africa) used were:

pat-left: 5' – ATGTCTCCGGAGAGGAGACCAGTTGAG - 3'

pat-right: 5' – CTCAGATCTGGGTAACTGGCCTAACTGG - 3'.

Reaction conditions were 35 cycles in 10 µl reaction volumes with 72 °C annealing temperature using *Taq* DNA polymerase (Promega, Madison, WI, USA), 0.5 µM primers and 1.5 µM $MgCl_2$. PCR products were resolved on a 1% agarose gel in TAE electrophoresis buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8,0) and visualised with UV light after staining with ethidium bromide (10 µg/ml).

5.3.3 ELISA with PAT antibodies

Non-transformed and PPT-resistant plants, two from the USDA (A5 and A6) and 10 from the UCT (T1 and nine clones of it) were analysed for expression of the PAT enzyme using a PAT-ELISA kit (Steffens Biotechnische Analysen, Ebringen, Germany).

Protein extraction. Soluble proteins were extracted from one leaf of each plant, cultured *in vitro*. Leaves were homogenised in extraction buffer supplied with the kit (5 ml/g) and the total soluble protein concentration of each sample was derived from a standard curve illustrated in Figure 10 according to Bradford (1976; Biorad, Hercules, CA, USA).

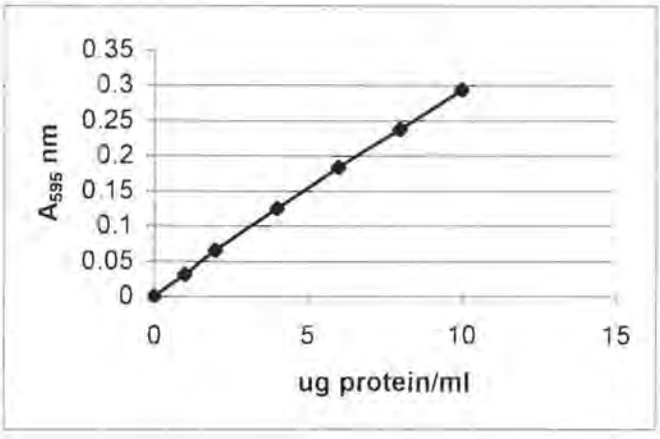


Figure 10 Standard curve that was used to determine the protein concentrations of leaf samples analysed with PAT-ELISA

ELISA method. Protein extracts (100 µl of each) were transferred to microwells containing immobilised PAT antibodies, incubated for 30 min at room temperature with vigorous shaking and washed five times with the wash buffer supplied with the kit. Diluted conjugate was added (100 µl per well), incubated and washed as above. Substrate (100 µl per well) was added followed by incubation for 30 min. Stop solution (100 µl per well) was added and the optical density determined at 650 nm with a spectrophotometer (Beckman, Palo Alto, CA, USA).

The amount of PAT expressed in each plant was calculated by comparison with standards supplied with the kit (0, 0.66, 1.8 and 4.9 ng PAT/ml standards, Figure 11). Each reaction was done in duplicate on two separate protein extracts.

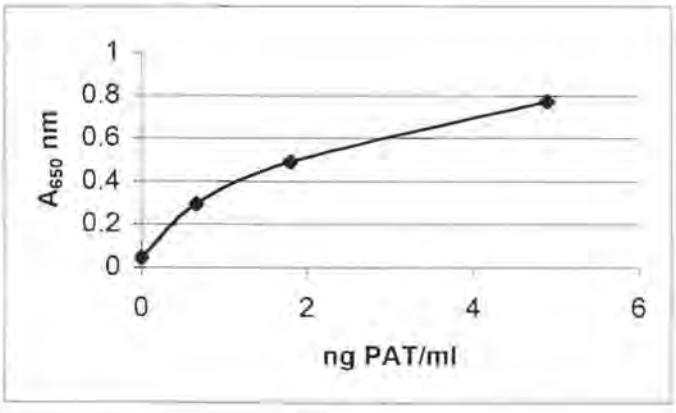


Figure 11 Standard curve used to determine the amount of PAT enzyme in leaf extracts analysed by ELISA

5.3.4 Southern blot analyses

DNA extraction. Genomic DNA from ten plants, five from a greenhouse (non-transformed, C6, C7, C13 and C14) and five cultured *in vitro* (non-transformed, C1, C2, C20 and C22) were analysed with Southern blots. DNA was isolated from leaves according to a modification of the method of Dellaporta *et al.* (1983). Individual samples (5 g) were ground to a fine powder in liquid nitrogen, resuspended in 40 ml extraction buffer (100 mM Tris, pH 8.0, 0.5 M NaCl, 50 mM EDTA and 0,07% (v/v) ß-mercaptoethanol) and 5 ml SDS (20% (w/v)). Samples were incubated for at least 30 min at 60 °C, 12 ml KOAc (5 M) was added followed by 20 min incubation on ice. Cell debris was sedimented by centrifugation (20 min at 22 000 x g at 4 °C) and the supernatant poured through cheesecloth wetted with 20 ml isopropanol. DNA was precipitated for 30min at –20 °C and recovered by centrifugation (15 min at 22 000 x g at 4 °C), resuspended in 2 ml TE buffer and treated with RNase A (150 µg/ml) for 30 min at 37 °C. The DNA suspension was extracted twice with an equal volume of phenol:chloroform (1:1), precipitated with 0.1 volume of 3 M NaOAc (pH 5.2) and 2 volumes of absolute ethanol, washed twice with 70% ethanol, dried and resuspended in TE buffer.

DNA electrophoresis and transfer. Two forms of DNA were resolved prior to hybridisation – uncut and digested with restriction enzymes. Aliquots containing 20 µg genomic DNA were digested overnight with either *Bgl*II or *EcoR*I (10 units/µg

DNA) restriction enzymes, and the products were resolved at 25 V overnight on a 0.8% (w/v) agarose gel in TAE electrophoresis buffer. DNA was transferred by capillary transfer to a Magnacharge nylon membrane (Micron Separations Inc., Westborough, MA, USA) according to Sambrook *et al.* (1989) with 20 x SSC (3 M NaCl, 0.8 M sodium citrate, pH 7.0). DNA was fixed to the membrane by baking at 80 °C for 1 h.

Digested genomic DNA was spiked with various amounts of plasmid p35SAC DNA to determine if the labelled probe could detect single copies of the *pat* transgene in 20 µg of genomic DNA background. p35SAC digested with *EcoR*I was electrophoresed alone or in combination with digested genomic DNA extracted from either greenhouse or tissue culture derived leaves. The amounts of plasmid DNA, equivalent to 0 (0 pg), 1 (5.5 pg), 2 (11 pg), 5 (28 pg) and 10 (55 pg) copies of the *pat* gene in 20 µg genomic DNA, were calculated according to the report of Nayak and Sen (1991) that the haploid genome of *O. thyrsoides* is 16.3 pg.

Copy numbers were determined as:

Size of 2n *O. thyrsoides* genome = (mass of genome)/(mass kb DNA)
$= (2x1.63x10^{-11})/(3.7x10^{4})(6.23x10^{-23})$
$= 3.26/2.3x10^{-18}$
$= \mathbf{1.39x10^{7}\ kb}$

Number of kb in 20 µg DNA = 20 µg/(mass of kb)
$= (20x10^{6}g)/(2.3x10^{-18}g)$
$= \mathbf{8.7x10^{12}}$

Number of copies of genome in 20 µg = (# of kb in 20 µg)/(# kb in genome)
$= 8.7x10^{12}/1.39x10^{7}$
$= \mathbf{6.26x10^{5}\ copies}$

p35SAC = 3.99 kb ∴ $6.26x10^{5}$ copies $= (6.26x10^{5})(3.99 \times 2.3x10^{-18})$
$= 5.7x10^{-12}$ g
$= \mathbf{5.7\ pg}$

The equivalent of 5 copies (28 pg) of the *pat* gene, represented by digested p35SAC DNA, was included in all blots containing DNA from transgenic plants.

Probe labelling and hybridisation. A ^{32}P-labelled *pat* gene probe was prepared by random prime labelling (Megaprime Random Prime Labelling Kit, Amersham, Buckinghamshire, England) of a 1.3-kb *EcoR*I fragment from p35SAC, including the CaMV35S promoter, *pat* gene and CaMV35S terminator (Appendix 1). The fragment was purified from an agarose gel prior to labelling using GELase (Epicentre Technologies, Madison, WI, USA). ^{32}P-dCTP with the highest specific activity available (6000 Ci/mmol; Amersham) was used to detect single copy integration events. Probes were labelled for an hour rather than the ten minutes prescribed by the protocol and reactions were not terminated with EDTA. Unincorporated nucleotides were removed with spin columns (Boehringer Mannheim, Mannheim, Germany). Denatured probe, labelled to 1 x 10^7 cpm was added to each membrane. Hybridisation was carried out for 16 h at 42 °C in 50% (v/v) formamide, 5 x Denhardt's solution, 0.5% (w/v) SDS and 100 µg/ml salmon testes DNA (Sigma, Saint Louis, MI, USA). Membranes were washed twice at room temperature and twice at 65 °C with 2 x SSC, 0.5% (w/v) SDS for 15 min to remove unbound probe. The section of membrane containing the DNA size marker (phage λ DNA digested with *Sty*I) was removed after transfer and hybridised separately with ^{32}P-labeled λ *Sty*I DNA. The membrane was exposed to X-ray film (Hyperfilm MP, Amersham) at –70 °C with intensifying screens for four to 18 days.

Probes (*pat* and λ *Sty*I molecular weight marker) could be reused on different blots on two consecutive days. Membranes containing the marker lanes were removed from the autoradiography cassette within 24 h to prevent overexposure and to allow genomic hybridisation signals to develop fully.

5.3.5 Northern-blot analyses

RNA extraction. Total RNA was extracted from either tissue culture- or greenhouse grown plants according to the method of Chomczynski and Sacchi (1987) as adapted by Colonna-Romano *et al.* (1998). Chemicals used were the best quality available, preferably tested RNase free. Glassware and consumables were treated overnight with 0.05% (v/v) DEPC in H_2O, autoclaved twice for 30 min and dried at 60 °C. Leaf samples (0.1 to 2 g) were ground to a fine powder in liquid nitrogen with a mortar and pestle, transferred to 4.5 ml GTC solution (4 M GTC, 25 mM Na citrate, 0.5% (w/v) sarkocyl, 0.1 M ß-mercaptoethanol) and mixed well. NaOAc (2 M, pH 4.0; 0.5 ml), phenol (pH 4.3, 4.5 ml) and chloroform:isoamylalcohol (24:1; 1 ml) were added, mixed thoroughly and incubated on ice for 15 min. Phases were separated by centrifugation (20 min at 12 000 x g; 4 °C) and the top aqueous phase was transferred to a clean tube. RNA was precipitated with an equal volume of isopropanol and the nucleic acid transferred to a clean tube with a sterile micro-pipette. The pellet was resuspended in 2 ml GTC solution with vigorous mixing and heating to 56 °C. RNA was precipitated with an equal volume of isopropanol, washed twice with 70% ethanol, dried slightly and resuspended in DEPC-treated H_2O. RNA concentrations and purity were determined with a spectrophotometer at 260- and 280 nm. A reading of $A_{260\ nm}$ = 1, represented 40 µg/ml RNA, therefore concentrations were calculated as

$$[\text{RNA}] \text{ in } \mu g/ml = A_{260\ nm} \times \text{dilution factor} \times 40$$

RNA electrophoresis and transfer. RNA integrity was determined with native agarose gel electrophoresis of 1 µg quantities in TAE buffer followed by staining with ethidium bromide (10 µg/ml). Prior to electrophoresis, all equipment was soaked in 0.1 M NaOH, 1 mM EDTA for 10 min and rinsed with DEPC-treated H_2O. Intact RNA was stored at –20 °C until required for denaturing electrophoresis for northern blots.

For northern analysis, RNA classes were separated under denaturing conditions for 2 to 3 h at 70 V in a 1 cm thick agarose gel (1.2% (w/v), 2% (v/v) formaldehyde, 0.2 M MOPS, 50 mM Na acetate, 10 mM EDTA) in MOPS running buffer. RNA samples (10 µg) were denatured in 4 volumes sample buffer (62.5% (v/v) formamide, 7.6%

(v/v) fromaldehyde, 1.25 x MOPS, 0.01% (w/v) bromophenol blue) for 10 min at 65 °C and transferred to ice. Separated RNA bands were visualised with ethidium bromide, transferred to a Magnacharge nylon membrane according to Sambrook *et al.* (1989) with 20 x SSC and fixed by baking at 80 °C for 1 h. DIG-labelled RNA molecular weight marker III (100 ng, Boehringer Mannheim) was included on each gel.

Probe labelling and hybridisation. Probes were prepared with random prime labelling (^{32}P-dCTP, 6000 Ci/mmol; Amersham Megaprime Kit, Amersham) of first, a 600-bp *Sal*I fragment from p35SAC containing the *pat* gene and second, a 1.8-kb *Eco*RI fragment from pJAM293 containing the *Antirrhinum majus* (snapdragon) polyubiquitin (*ubi*) gene fragment from pJAM392 (gift from Dan Bergey, Genbank accession number X67957). Fragments were purified from agarose prior to labelling using GENECLEAN (BIO101, Vista, CA, USA). Denatured probe, (4.6 x 10^6 cpm of either *pat* or *ubi*) was added to each membrane and hybridised for 16 h at 52 °C in hybridisation solution (0.625 M $NaPO_4$, 1.25% (w/v) SDS, 1.25 mM EDTA, pH 7.2). Membranes were washed (3 x 15 min at 52 °C in 0.1 x hybridisation solution) to remove unbound probe and exposed to X-ray film (Hyperfilm MP, Amersham) at –70 °C with intensifying screens for one to five days.

Stripping and reprobing. Bound *pat* probe was stripped from membranes by adding boiling 0.5 % (w/v) SDS. The membrane was incubated on an orbital shaker until the SDS had cooled to room temperature after which the wash was repeated. Membranes were hybridised for a second time with a ^{32}P-labelled 1.8-kb *Eco*RI fragment containing the *ubi* gene, used as a positive internal control. Membranes were exposed to X-ray film with intensifying screens at –70 °C for one to four days.

Detection of DIG-labelled molecular weight marker. The lane containing the DIG-labelled RNA molecular weight marker was cut from each membrane prior to hybridisation and visualised using a fluorescent DIG detection kit (Boehringer Mannheim). The membrane was incubated with blocking reagent for 1 h, with anti-DIG antibody conjugate (1:10 000 in blocking reagent) for 30 min, washed 3 x 15 min

with wash buffer, equilibrated with detection buffer and visualised on an X-ray film (Hyperfilm, Amersham) with CSPD fluorescing substrate (Boehringer Mannheim). The developed autoradiograph was aligned with the ones containing total RNA hybridised to the *pat*- and *ubi* genes.

5.3.6 Greenhouse trials

Acclimatisation of in vitro plants. Non-transformed and PPT-resistant plants that were two- to five-cm tall and propagated *in vitro* from leaf explants of plants from the USDA- and the UCT experiments were hardened off and planted in a greenhouse. The roots were washed thoroughly in water to remove all traces of agar and medium prior to planting in 12 cm pots without saucers in soil:peat moss (1:1), in a greenhouse (18/26 °C). Plants were watered daily for two weeks and on every second day thereafter. Fertiliser was applied every four weeks for the first six months after hardening off.

Optimisation of leaf paint assay. Herbicide tolerance of greenhouse-grown plants was tested with six different concentrations of Ignite® (0 (0 mM), 1 (10 mM), 3 (30 mM), 5 (50 mM), 10 (100 mM) and 20% (200 mM PPT)). Each plant tested was painted with only one concentration of PPT. One leaf per plant was painted from the base to the tip on both adaxial and abaxial surfaces with cotton wool dipped in the appropriate Ignite® solution, preventing herbicide from running into the bulb. There were four replicates per concentration and results were taken after 14 days.

Leaf paint assay on transgenic plants. PPT-resistant plants, both from the USDA and the UCT were painted with 30 mM PPT (0.3% (v/v) Ignite®) after six months cultivation in a greenhouse. For the USDA study, one non-transformed and ten PPT-resistant events (lines A1 – A10; 3 to 20 plants per line) were tested. For the UCT study, 13 clones of T1 were tested. One to two leaves per plant of both non-transformed and PPT-resistant plants were painted, depending on the number of leaves available. Results were taken after 14 days.

5.4 Results

5.4.1 *In vitro* propagation of PPT-resistant plants

From the USDA study, 135 individual plants had regenerated from bombarded callus and formed roots within one year on 15 µM PPT. Leaf explants from the ten strongest plants, designated A1 to A10, could be propagated on 5 µM PPT and seven to 30 clones regenerated from each line. However, only three plants (A1, A5 and A9) performed visibly better than non-transformed control leaf explants cultured on the same medium and regeneration frequencies were low compared to the regeneration control (results not shown).

Tissues bombarded at the UCT were cultured on 20 µM PPT and 80 rooted plants regenerated from traditional biolistics and 33 from the Taxi method. After six months, three plants from the Taxi experiments were taller than 5 cm but due to the stricter selection, only one plant, designated T1, survived long enough to allow propagation. In contrast to results from the USDA, 37 clones regenerated on 7.5 µM PPT whilst all non-transformed leaf explants died as illustrated in Figure 12.

5.4.2 PCR analysis for the presence of the *pat* gene

Within six months after bombardment, ten plants from the USDA (A1 to A10) tested positive with PCR as illustrated in Figure 13. During the next six months, 32 more plants were analysed. Twenty-nine tested positive for the presence of the *pat* gene but the results could not be repeated for separate DNA extracts or for individual PCR replicates (results not shown).

Figure 12 Regeneration from leaf explants of (**a**) a non transformed plant (left) and T1 (right) cultured on 7.5 µM PPT for four weeks and (**b**) survival and regeneration from leaves of T1 clones reintroduced into tissue culture after three months in a greenhouse. Leaf explants were cultured on 7.5 µM PPT for three weeks from (1) C2, (2) C6, (3) C7, (4) C10, (5) C12, (6) C13, (7) C14, (8) C16, (9) C20 and (10) C22

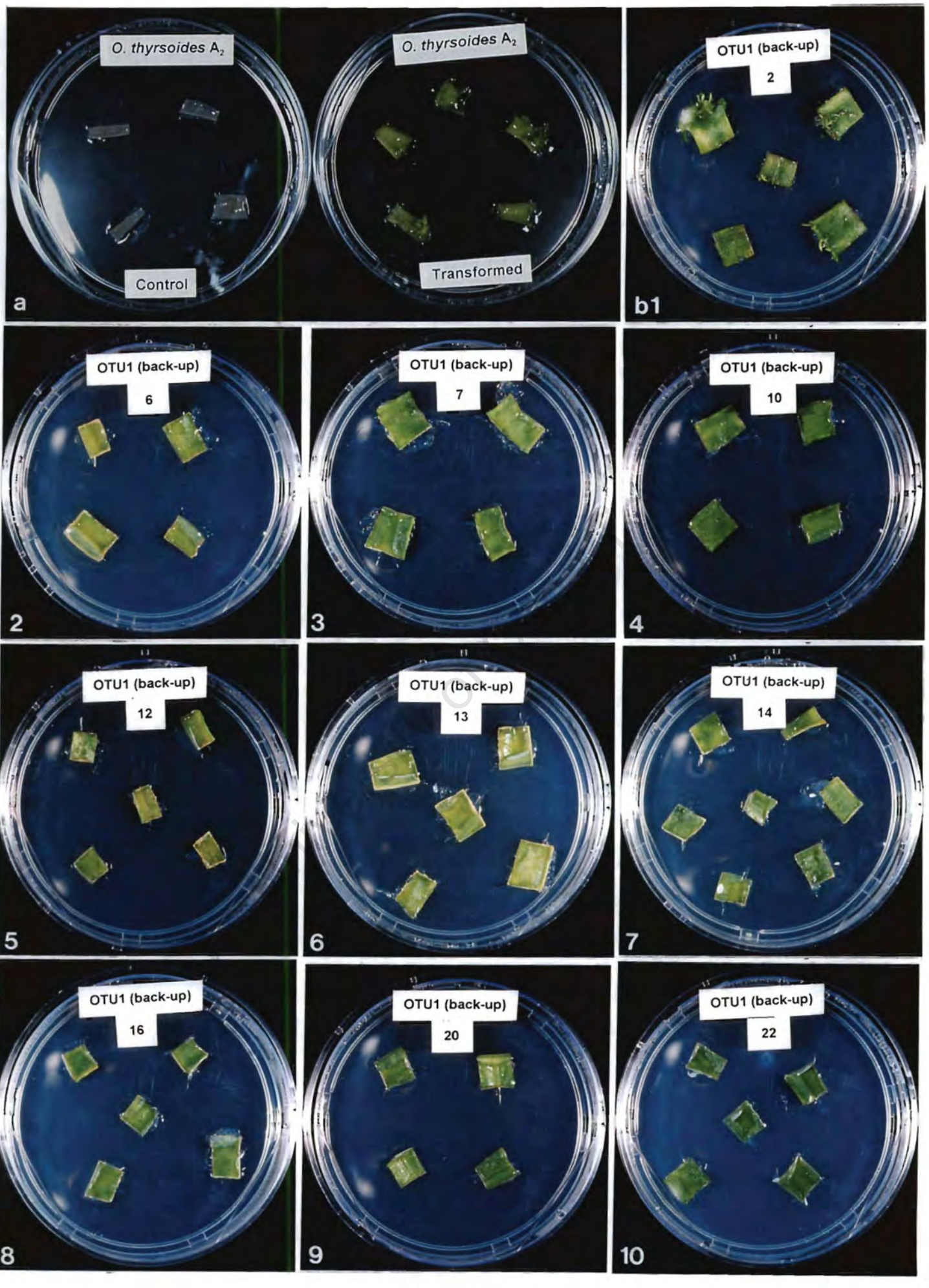

O. thyrsoides A2
O. thyrsoides A2
Control
Transformed
a
OTU1 (back-up)
2
b1
OTU1 (back-up)
6
2
OTU1 (back-up)
7
3
OTU1 (back-up)
10
4
OTU1 (back-up)
12
5
OTU1 (back-up)
13
6
OTU1 (back-up)
14
7
OTU1 (back-up)
16
8
OTU1 (back-up)
20
9
OTU1 (back-up)
22
10

Figure 12

On the other hand, only three plants that regenerated from Taxi experiments performed at the UCT, illustrated in Figure 14, were large enough for PCR analysis within six months after bombardment. They were designated T1, T2 and T3. Figure 15a, lane 3 shows that T1 gave a strong signal, T3 a faint signal (lane 5) and T2 no detectable signal (lane 4). Five clones that subsequently regenerated from leaf explants of T1 - C6, C7, C12, C13 and C14 - consistently showed the expected band in PCR analyses of three separate DNA extractions, illustrated in Figure 15b, c and d.

Figure 13 Plants that regenerated on selection medium from callus bombarded in the UCT study were designated, from left to right, T1, T2 and T3, respectively and were analysed by PCR.

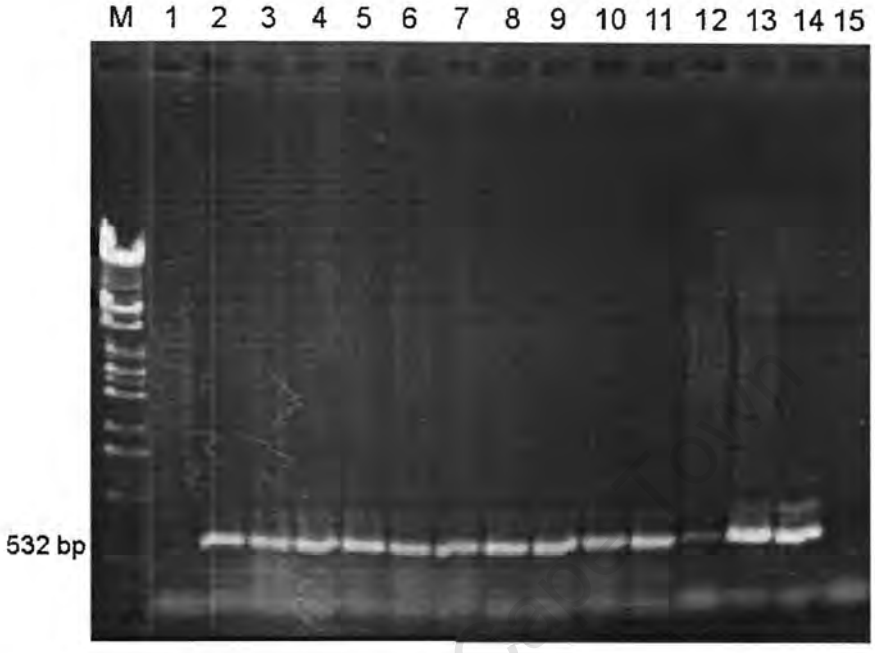


Figure 14 Result of PCR analysis for the presence of the *pat* gene of the ten strongest plants from the USDA. Lanes represent: (M) λ *Sty*I molecular weight marker; PCR products from (1) a non-transformed plant; (2) to (11) USDA events A1 to A10; plasmid p35SAC DNA equivalent to (12) 1 copy of the *pat* gene; (13) 10 copies of the *pat* gene; (14) 100 copies of the *pat* gene and (15) H_2O control.

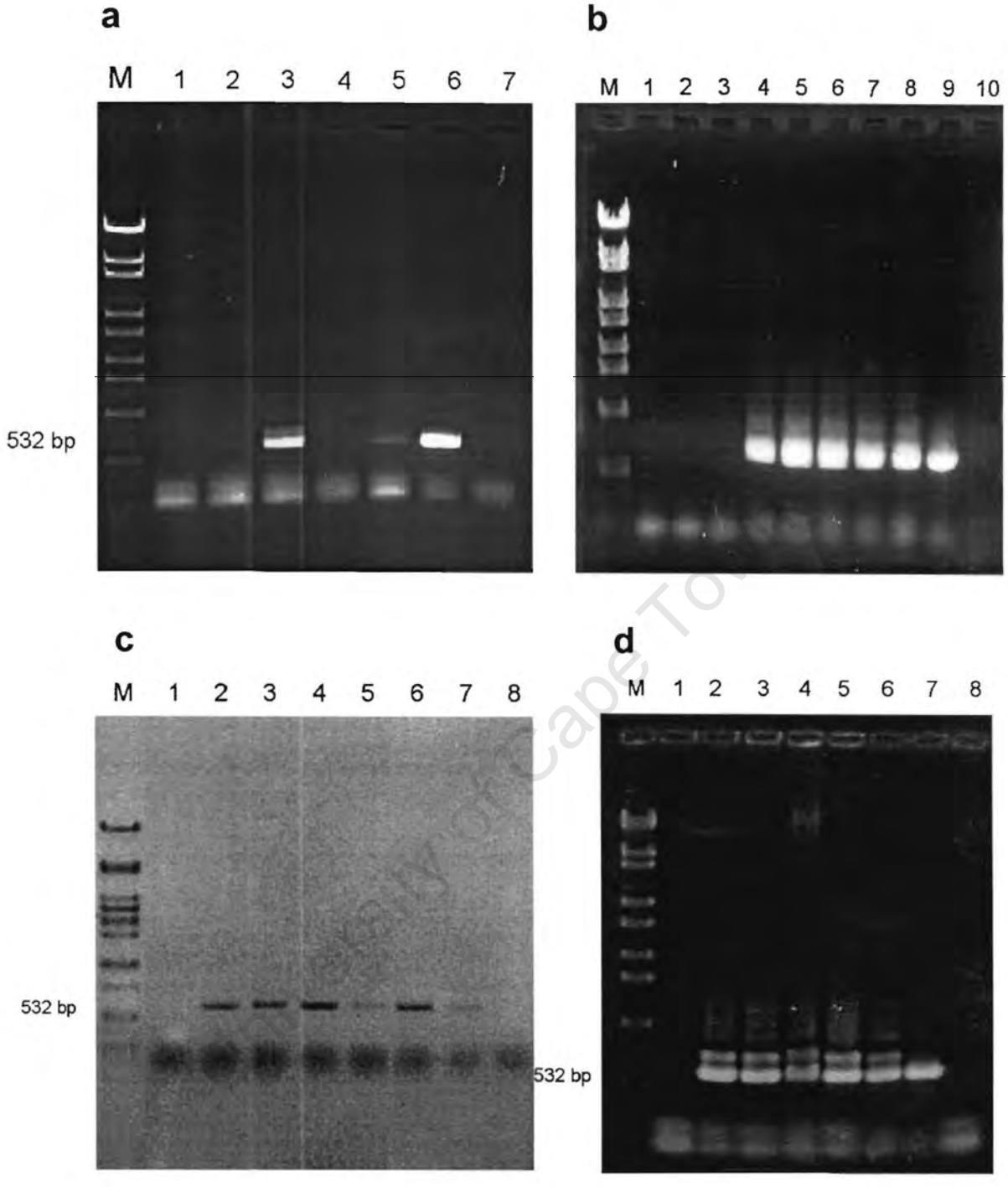


Figure 15 **(a)** PCR analysis of T1 plants. Lanes: (M) λ *Sty*I molecular weight marker; (1) and (2) non transformed plants; (3) T1; (4) T 2; (5) T 3; (6) plasmid control (7) H_2O.
(b) PCR analysis of clones of T1. Lanes: (M) λ *Sty*I molecular weight marker; (1), (2) and (3) non transformed plants; (4) C6; (5) C7; (6) C12; (7) C13; (8) C14; (9) plasmid control (10) H_2O.
(c) – **(d)** PCR analysis of clones of T1. Lanes: (M) λ *Sty*I molecular weight marker; (1), non transformed plant; (2) C6; (3) C7; (4) C12; (5) C13; (6) C14; (7) plasmid control (8) H_2O.

5.4.3 ELISA with PAT antibodies

One leaf from two plants form the USDA (A5 and A6), T1 from the UCT, nine clones from T1 (C2, C6, C7, C10, C13, C14, C16, C20 and C23) and two non-transformed control plants were assayed with a PAT-ELISA. Table 10 shows that T1 and all its clones expressed the PAT protein but none of the USDA plants did. The level of expression varied; six plants, C2, C7, C10, C16, C20 and C23, expressed more PAT than T1, whereas C6, C13 and C14 expressed less. A typical result for A5, A6, T1 and a non-transformed control is illustrated in Figure 16.

Figure 16 ELISA with antibodies directed against the PAT enzyme. The wells contained, from left to right, four standards (0, 0.66, 1.8 and 4.9 ng PAT/ml) and protein extracts from T1, A5, A6 and a non-transformed plant, respectively.

Results from the PCR and ELISA analyses of plants from the USDA study were ambiguous and these plants were not analysed further. At this point, the event derived from UCT, designated T1, was sacrificed for propagation and only clones of T1 were analysed with Southern and northern blots.

5.4.4 Southern blot analyses

Isolation of DNA was difficult but sufficient quantities were obtained for restriction digests with *EcoR*I and *Bgl*II for Southern analyses. DNA suspensions, especially those from greenhouse plants, were generally viscous and the yields were low, typically 15 to 40 µg/g fresh leaf tissue. Ageing greenhouse plants and plants grown in the dark for 4 to 7 days prior to DNA isolation yielded better quality DNA.

Table 10 PAT expression in putative transgenic PPT-resistant plants obtained from the USDA and UCT studies as determined with ELISA. Values are the means from two separate assays, each containing two replicates per sample.

Sample[a]	Absorbance at $_{650nm}$	Amount of PAT expressed	
		ng/ml protein extract	ng/g fresh leaf
Control	0.041 ± 0.020	0	0
A5[a]	0.037 ± 0.008	0	0
A6	0.045 ± 0.012	0	0
T1[b]	0.301 ± 0.014	0.394	1.76
C2[c]	0.690 ± 0.003	0.970	4.85
C66	0.076 ± 0.010	0.050	0.42
C7	0.244 ± 0.013	0.305	2.54
C10	0.297 ± 0.008	0.380	4.04
C13	0.108 ± 0.012	0.100	1.38
C14	0.090 ± 0.013	0.070	0.97
C16	0.330 ± 0.027	0.430	2.15
C20	0.192 ± 0.029	0.225	2.56
C23	0.183 ± 0.033	0.210	2.56

[a] plants from the USDA study
[b] plants from the UCT study
[c] clones of T1

When the sensitivity of the hybridisation reaction was determined, faint signals were visible for p35SAC DNA representing one copy of the *pat* gene included in 20 µg genomic DNA (Figure 17, lanes 3 and 12) and strong signals appeared for five and ten copies (lanes 4, 5, 14 and 15). Signal strengths for greenhouse and tissue culture derived DNA were comparable but when only plasmid DNA was run on the gel (lanes 6, 7, 8 and 9) the signals were less pronounced.

Agarose gels of DNA from PPT-resistant plants showed that uncut genomic DNA resolved as smears in the high molecular weight region of an agarose gel, with one predominant band of approximately 19 kb, as illustrated in Figure 18a, lanes 1 to 4. The Southern blot of this gel is illustrated in Figure 18b and shows that probe binding to viscous DNA samples are often inefficient (lanes 2 and 3). It was also found that these viscous samples cut poorly with restriction enzymes (results not shown). Frequent mixing improved digestion, resulting in even smears on a gel (Figure 18a, lanes 5 to 8 and 10 to 12).

Figure 18b further illustrates that the *pat* gene hybridised to uncut DNA from all three T1 clones tested (C6 in lane 2, C7 in lane 3 and C14 in lane 4). No hybridisation signals were observed with DNA from a non-transformed plant (lane 1). The bands appeared in the high molecular weight region larger than 15 kb, indicating that the *pat* gene had integrated into the genomic DNA of all the clones although the signal for C7 in lane 3 was much fainter than those for C6 and C14 in lanes 2 and 4.

In Southern blots of DNA digested with *EcoR*I the *pat* gene probe hybridised with DNA from all T1 clones tested, cultured either in a greenhouse (Figure 18b, lanes 5 to 8) or in tissue culture (Figure 19, lanes 2 and 3). Hybridisation occurred predominantly in the 1.3-kb region, although some larger bands of up to 4.5 kb were also visible. For DNA digested with *Bgl*II, one band of 4 kb appeared in all the T1 clones tested (Figure 18b, lanes 11 and 12 and Figure 19, lanes 6 to 9) as well a few larger bands comparable in size to uncut genomic DNA.

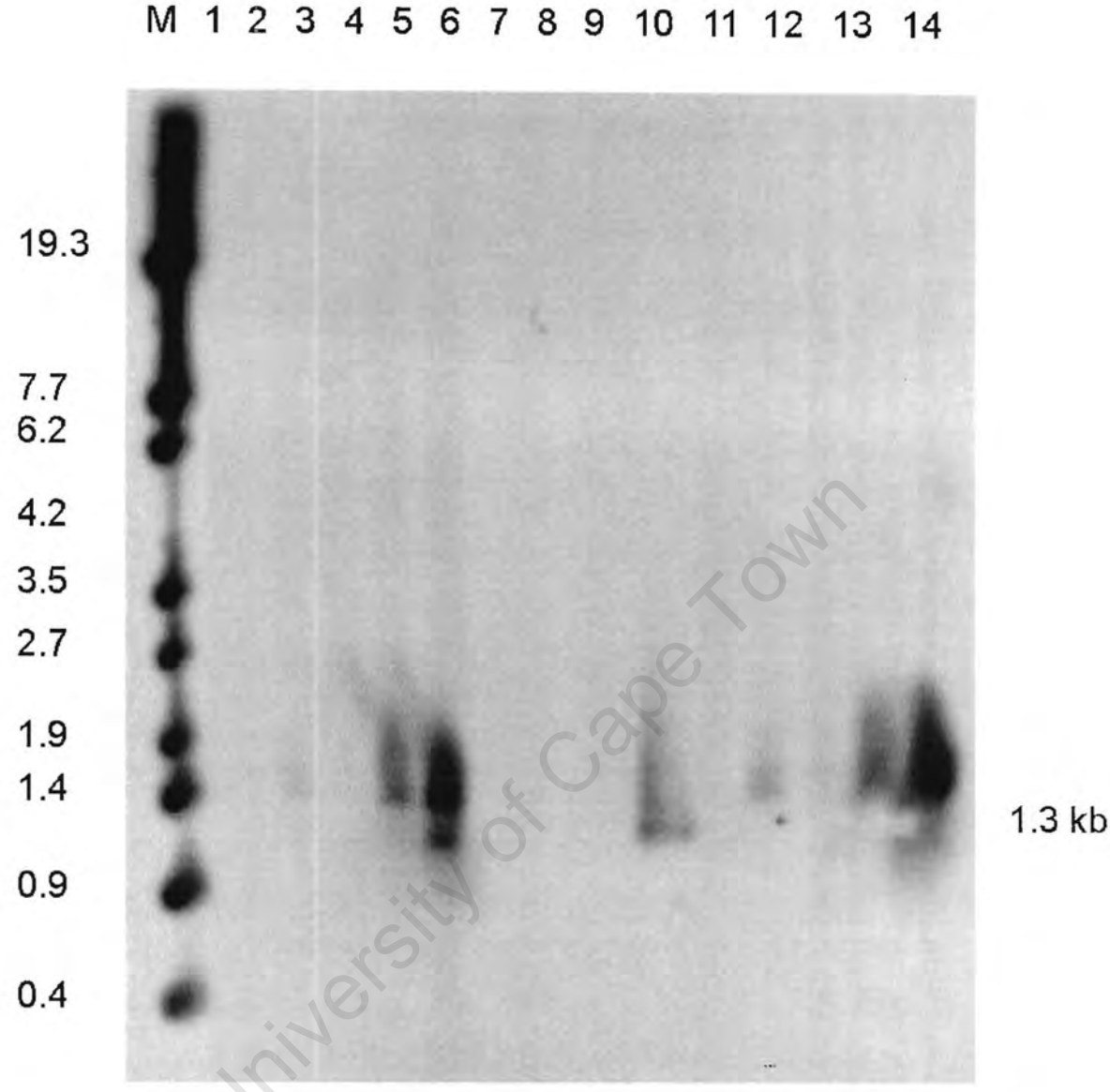


Figure 17 Southern blot of genomic DNA isolated from non transformed plants cultured in a greenhouse and *in vitro*, spiked with various copies of the *pat* gene (p35SAC) DNA. Lanes: (M) λ *Sty*I molecular weight markers, (1) – (5) greenhouse DNA spiked with 0, 1, 2, 5 and 10 copies of *pat*, respectively, (6) – (9) 1, 2, 5 and 10 copies of *pat* DNA, (10) - (14) tissue culture DNA spiked with 0, 1, 2, 5 and 10 copies of *pat*, respectively.

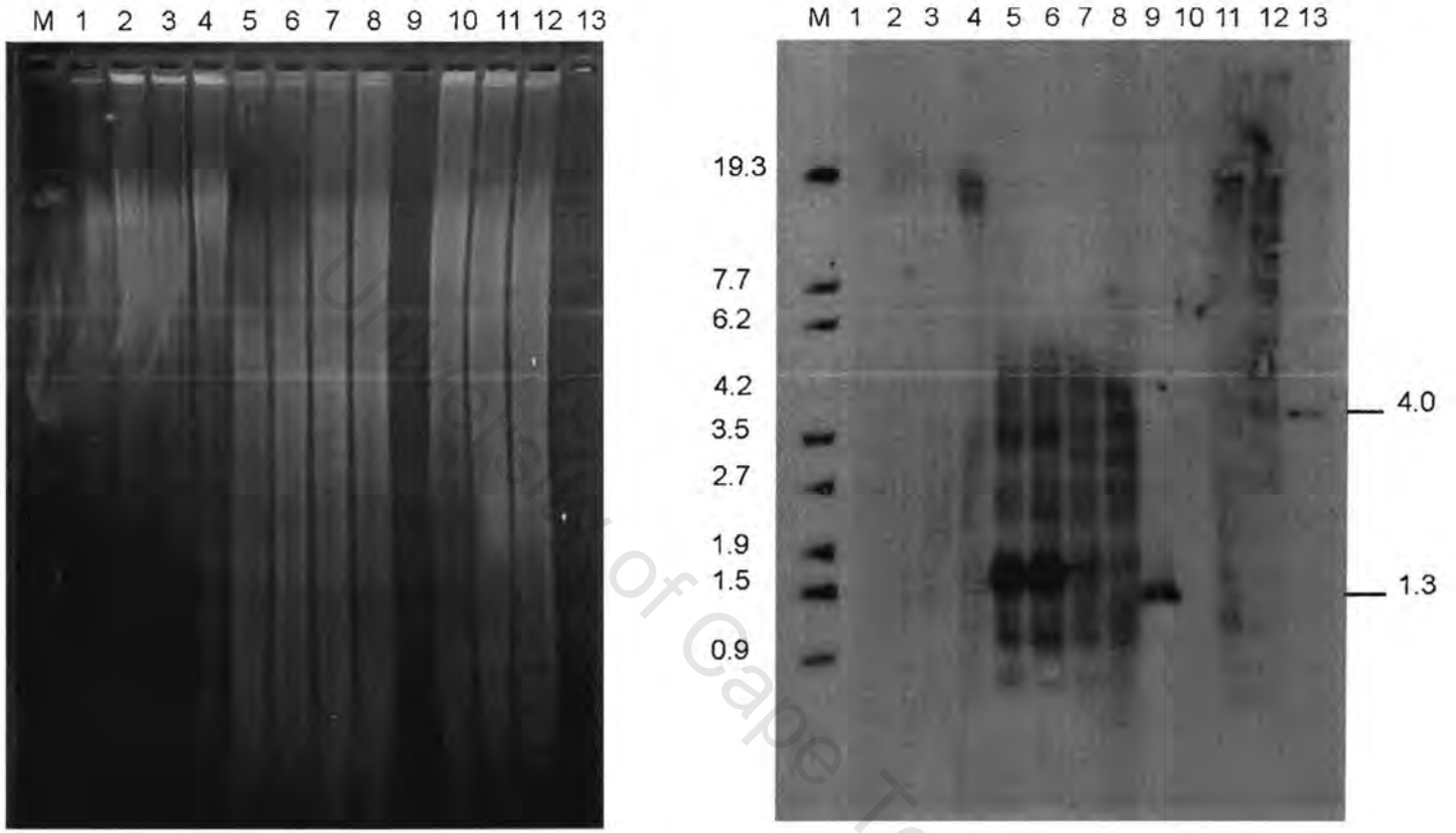


Figure 18 DNA isolated from greenhouse grown clones of T1, digested with restriction enzymes, (a) resolved on an agarose gel and (b) analysed for Southern hybridisation. Radioactively labelled *pat* gene (1.3kb) was used as probe. Lanes (1) - (4) contained 20 µg undigested genomic DNA of non transformed and three T1 clones, C6, C7 and C14, respectively. Lanes (5) - (8) contained 20 µg of genomic DNA of T1 clones C6, C7, C13 and C14 digested with *EcoR*I, respectively. Lanes (10) - (12) contained 20 µg of genomic DNA digested with Bg*l*II from a non transformed and two T1 clones C6 and C7, respectively. Lanes (9) and (13) contained 28 pg of p35SAC plasmid control DNA digested with *EcoR*I and *Bgl*II, respectively. Lane M contained λ*Sty*I DNA molecular marker probed separately with labelled marker. The sizes of marker fragments are shown on the left.

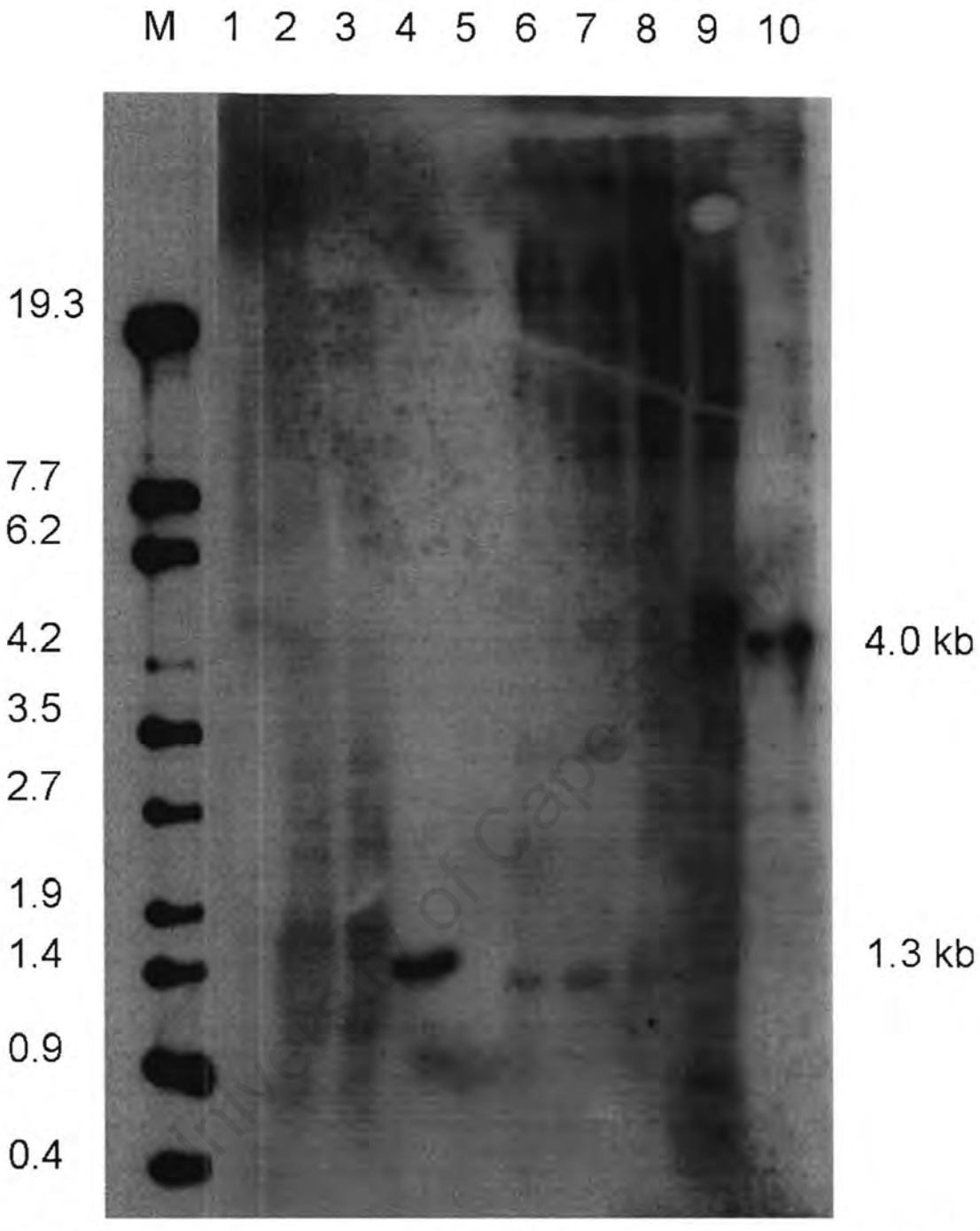


Figure 19 DNA isolated from *in vitro* cultured clones of T1, digested with restriction enzymes resolved on an agarose gel and analysed for Southern hybridisation. Radioactively labelled *pat* gene (1.3kb) was used as probe. Lanes (1) – (3) contained 20 µg of genomic DNA digested with *EcoR*I fro a non transformed plant and T1 clones C2 and C22, respectively. Lanes (5) – (9) contained 20 µg of genomic DNA digested with *Bgl*II from a non transformed plant and four T1 clones C1, C2, C20 and C22, respectively. Lanes (4) and (10) contained 28 pg of p35SAC plasmid control DNA digested with *EcoRI* and *Bgl*II, respectively. Lane M contained λ*Sty*I DNA molecular weight marker probed separately with labelled marker. The sizes of marker fragments are shown on the left.

5.4.5 Northern blot analyses

RNA extraction was also difficult. The method of Chomczynski and Sacchi (1987) was found to be fast and yielded superior RNA compared to that of Linda Walling (University of California, Riverside, CA, USA, pers. comm.). Polysaccharide contamination occurred in RNA obtained from greenhouse grown plants in seven separate sets of extractions and prevented resolution of bands under denaturing conditions (results not shown). Culturing plants in the dark did not help. Better results were obtained with leaves from plants cultured *in vitro* and RNA concentrations varied from 40 to 200 µg/g leaf tissue. All subsequent experiments were performed on RNA from plants cultured *in vitro*.

One non-transformed and seven T1 clones (C2, C6, C7, C12, C13, C14 and C16) were analysed and non-denaturing electrophoresis confirmed that the RNA from all eight plants were intact, although contaminating polysaccharides caused smears and complicated handling of samples as illustrated in Figure 20a. RNA that exhibited two bands in the high molecular weight region of the gel was subsequently used for denaturing electrophoresis and northern blots.

Denaturing electrophoresis of RNA from all eight plants revealed four bands and faint smears of degraded RNA after ethidium bromide staining as illustrated in Figure 20b. This RNA, immobilised on a nylon membrane, was hybridised sequentially with the *pat*- and *ubi* gene probes.

^{32}P-labelled *pat* hybridised predominantly with a band slightly larger than 1.5 kb in all T1 clones as illustrated by Figure 21a, lanes 3 to 9, but not with the non-transformed control in lane 2, indicating that only the transgenic plants expressed the gene. C12, C14 and C16 exhibited stronger bands than the other four plants. The *ubi* probe used as an internal standard to verify that an equal amount of RNA was loaded in each lane hybridised to all samples, including the non-transformed control. All plants exhibited signals of comparable strength as shown in Figure 21b except for C13 (lane 6), which showed a fainter signal, indicating that for this clone less RNA was loaded on the original gel.

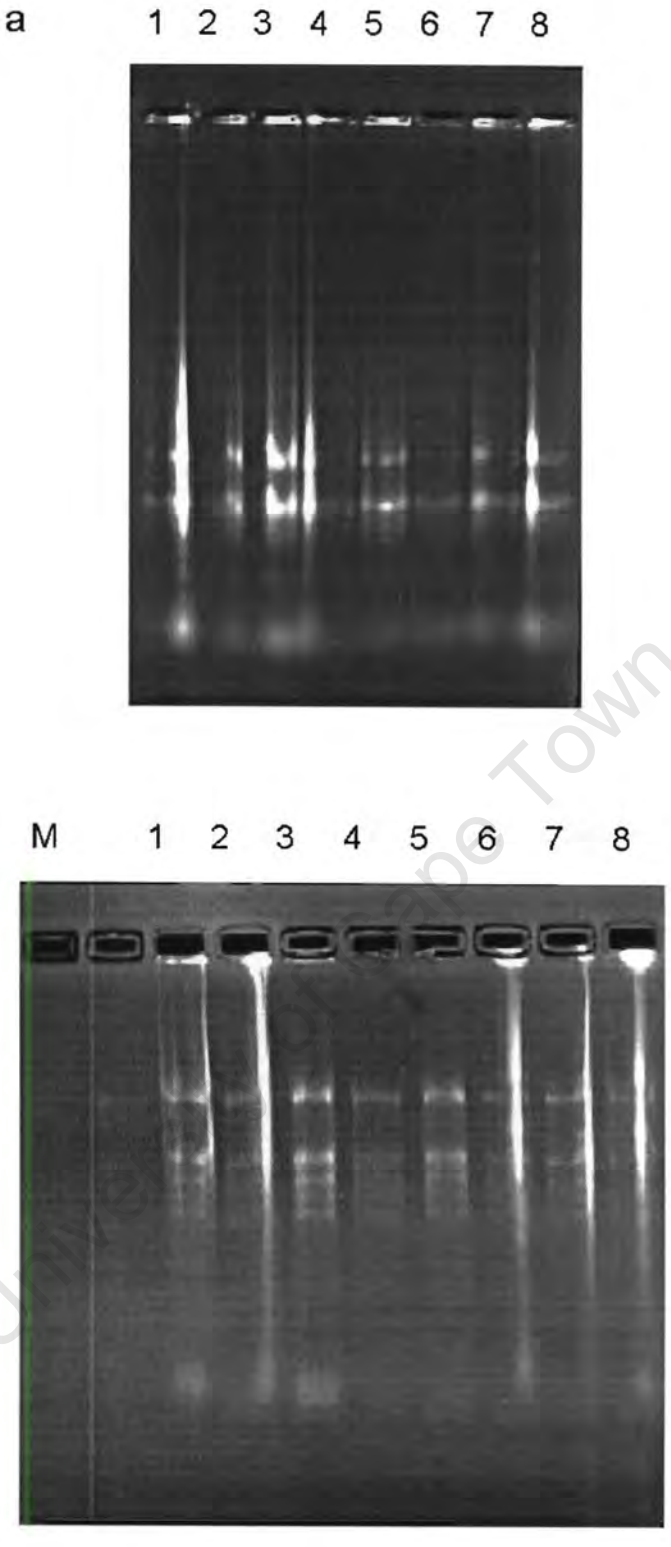


Figure 20 Total RNA isolated from plants cultured *in vitro* resolved on agaros gels under (a) non-denaturing and (b) denaturing conditions. Lanes (M) RNA molecular weight markers III, RNA from (1) a non transformed plant and T1 clones (2) C2, (3) C6, (4) C7, (5) C12, (6) C13, (7) C14 and (8) C16, respectively.

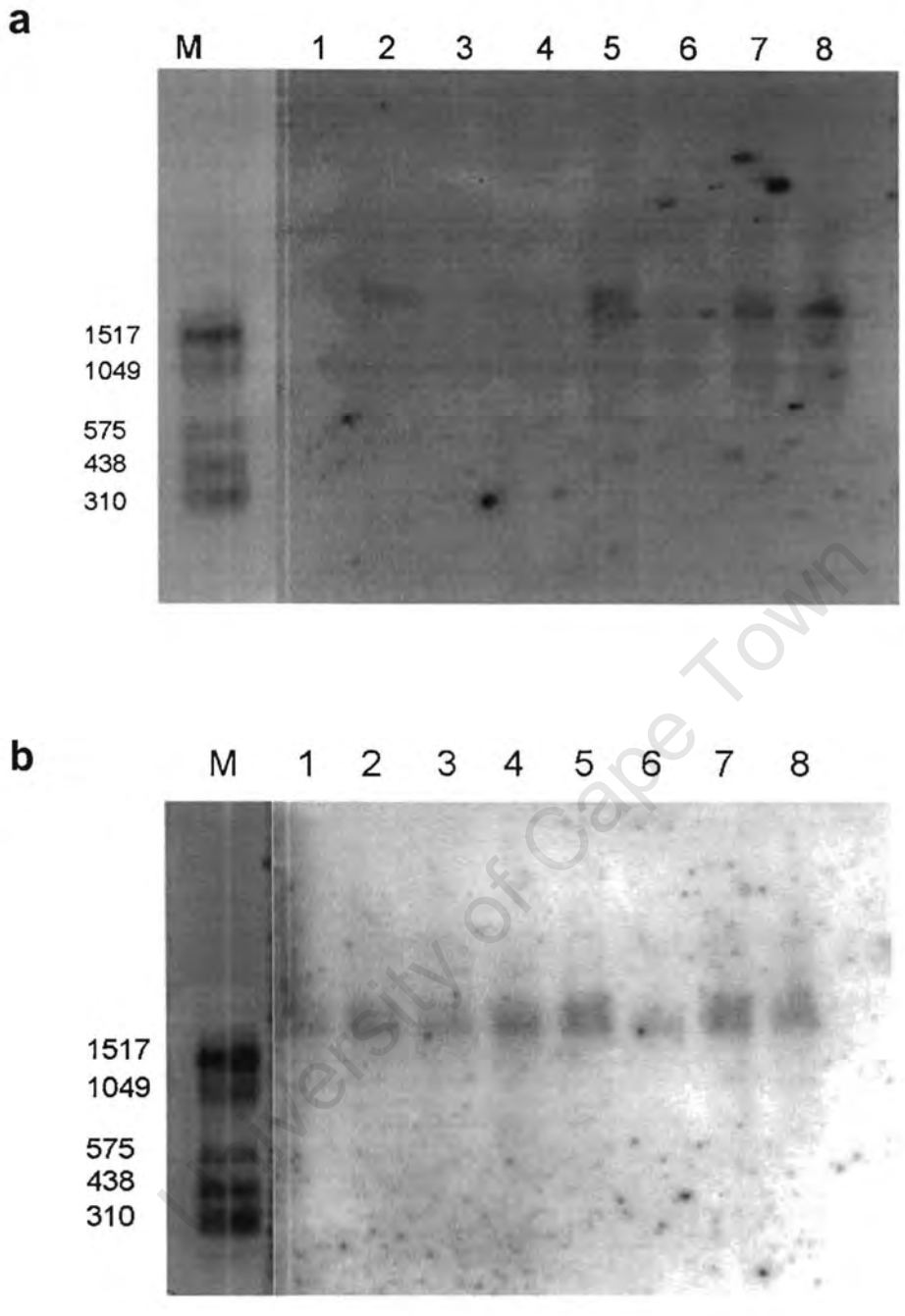


Figure 21 Northern blots with total RNA isolated from T 1 clones cultured *in vitro* and hybridised with (a) the *pat* gene and (b) an ubiquitin internal standard. Lanes: (M) RNA molecular weight marker III; (1) non transformed plant and (2) – (8) T1 clones C2, C6, C7, C12, C13, C14 and C16, respectively.

5.4.6 Greenhouse trials

Acclimatisation of in vitro plants. Non-transformed plants and 23 of the 37 clones of T1 were transferred from *in vitro* culture to a greenhouse. As Figure 22 shows, both non-transformed and transgenic plants appeared normal with regard to plant and flower morphology. After three months in the greenhouse, leaves from ten of these plants were reintroduced to tissue culture and could be propagated on 7.5 µM PPT, illustrated in Figure 12b.

Leaf paint assay. Optimisation of the leaf paint assay with different concentrations of the herbicide Ignite® indicated that a 3% (v/v) solution, equal to 30 mM PPT, caused severe damage to non-transformed leaves within 14 days after application (Figure 23a). Higher concentrations of PPT were lethal to whole plants, therefore 30 mM PPT was used to test for herbicide resistance in transgenic clones of T1.

Table 11 shows that the putative transgenic plants from the USDA (lines A1 – A10) were not more tolerant to PPT than the non-transformed controls. Table 12 represents the results of the UCT study where 13 clones of T1 were tested. Painted leaves from three clones - C7, C12 and C20 - remained undamaged whilst the leaves of all the non-transformed plants that were tested died within 14 days (Figures 23b and c). The remaining ten clones exhibited various degrees of tolerance as illustrated in Figure 24. Only three clones - C17, C18 and C22 - showed more than 25% damage of the painted leaf surface but it was still less than that observed for non-transformed controls.

The average score calculated for the USDA plants was 3.4 ± 0.7 and was essentially similar to the score of 3.2 ± 1.8 obtained for the non-transformed controls. In contrast, the average score for the 13 T1 clones was 1.0 ± 0.7 compared to 4.3 for the controls, indicating that the T1 clones were resistant to the herbicide but not the USDA plants.

Table 11 Leaf paint assay results from 10 lines of PPT-resistant plants obtained from the USDA study. Observations were made 14 days after one leaf of each plant was painted with 3 % (v/v) Ignite®, equal to 30 mM PPT and is presented as an average ± SD for each line. Scores allocated were 0 (no visible damage), 1 (slight discoloration or leaf tip died), 2 discoloration or damage to 25 % of leaf), 3 (painted surface discoloured yellow), 4 (whole leaf discoloured) or 5 (the painted leaf died).

Line	Number of plants tested per line	[a]Average score
Non-transformed	11	3.2 ± 1.8
A1	22	2.3 ± 1.4
A2	3	3.6 ± 1.5
A3	6	3.8 ± 1.3
A4	3	3.6 ± 2.3
A5	10	2.9 ± 1.5
A6	4	3.0 ± 0.9
A7	3	4.7 ± 0.6
A8	3	4.0 ± 1.7
A9	12	2.9 ± 1.9
A10	13	3.0 ± 1.4

Figure 22 In the greenhouse, *O. thyrsoides* A2 plants that were propagated from the transgenic line T1, exhibit normal leaf morphology with regard to (**a**) leaf shape and size in a randomised greenhouse trial where NT = non-transformed controls and UCT = T1 clones and (**b**) flower morphology of two T1 clones.

Figure 23 (Overleaf) (**a**) Optimisation of the leaf paint assay with Ignite® solutions containing decreasing concentrations of PPT from left to right, 200 mM, 100 mM, 50 mM, 20 mM, 10 mM, and 0 mM. Results were taken 14 days after application of the herbicide. (**b**) Effect of 30 mM PPT painted on leaves of three non-transformed control plants (left) and the three T1 clones (right) C7, C12 and C20. (**c**) Detail of the individual painted leaves in (b).

Figure 24 (Second overleaf) Varying degrees of herbicide resistance displayed by eight T1 clones (right) compared to non-transformed controls (left) in the leaf paint assay, (**a**) in the greenhouse and (**b**) on each of the painted leaves.

Figure 22

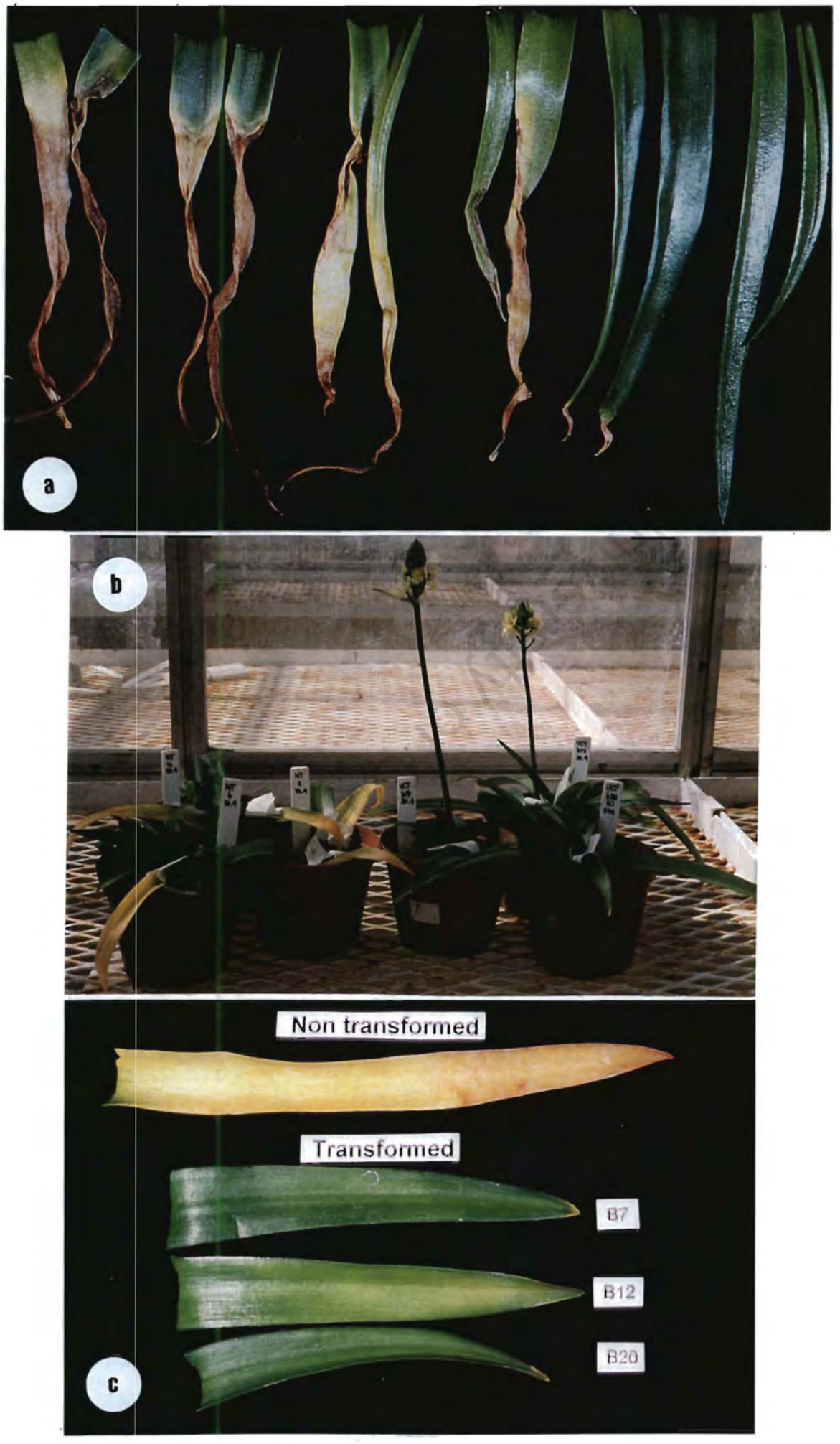


Figure 23

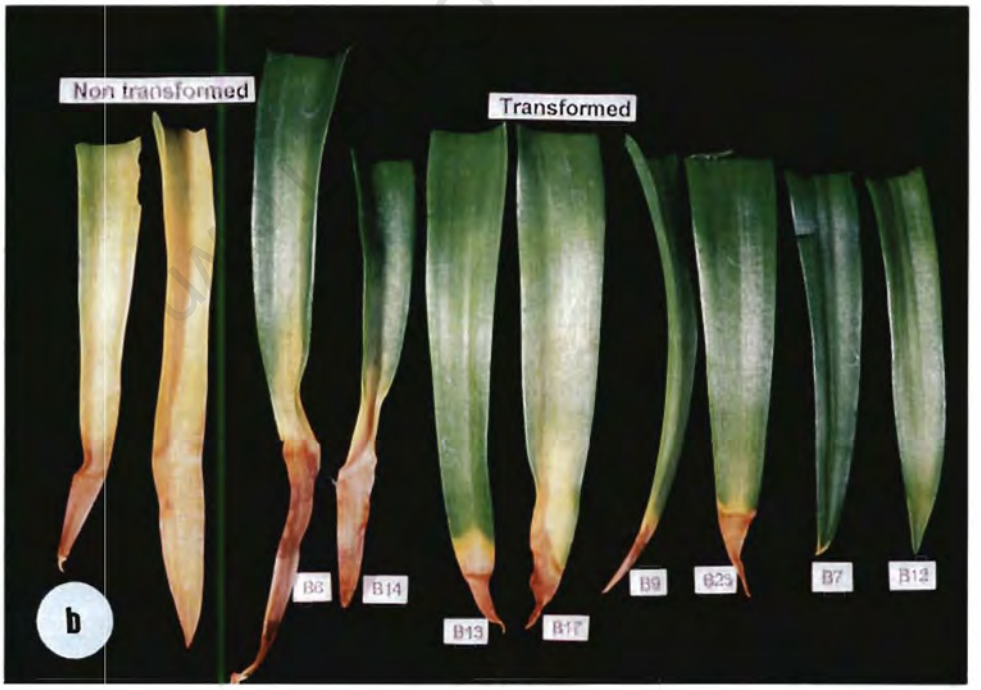


Figure 24

Table 12 Leaf paint assay results from 13 clones of the PPT-resistant transgenic plant, T1, obtained from the UCT study. Observations were made 14 days after leaves were painted with 3 % Ignite® (30 mM PPT). Scores allocated were 0 (no visible damage), 1 (slight discoloration or leaf tip died), 2 discoloration or damage to 25 % of leaf), 3 (painted surface discoloured yellow), 4 (whole leaf discoloured) or 5 (the painted leaf died).

T1 clones	No. of leaves painted per plant	Average score
Non-transformed (4 plants)	1, 1, 2 and 2	4.3
C6	1	1
C7	1	0
C9	2	1
C12	2	0
C13	1	1
C14	1	1
C17	2	2
C18	2	2
C19	2	1
C20	1	0
C21	1	1
C22	2	2
C23	1	1

5.5 Discussion

5.5.1 The USDA study

From the USDA study, 135 individual events regenerated from bombarded callus, indicating the presence of transformed tissues that could detoxify PPT and differentiate into plants. Propagation from leaf explants of ten events were possible on 5 µM PPT and from three events on 7.5 µM PPT. Surprisingly, rooted plants also regenerated from seven non-transformed explants on 5 µM PPT, indicating that regeneration of escapes are possible for *O. thyrsoides* A2 on selection medium.

Ambiguous results were also obtained with PCR analyses. It was first assumed to be due either to inefficient DNA isolation or interference of contaminating polysaccharides with the PCR reaction. To solve this problem, various DNA isolation protocols were tested, including the one described in materials and methods, CTAB protocols (Rogers and Bendich, 1994; Porebski *et al.*, 1997), sephadex purification (R. Griesbach, personal communication), differential precipitation with NaCl and EtOH (Fang *et al.*, 1992; Michaels *et al.*, 1994) and additional phenol extractions. None of these methods improved the consistency of the results. It is postulated that the plants analysed were chimeric, resulting in different PCR results from different DNA isolations which may have been performed on non-transformed and transformed sections of an individual plant on different occasions. This theory is supported by the results of Watad *et al.* (1998) who found that many escapes regenerated from bombarded callus of *Lilium*.

In addition, ELISA analysis of A5 and A6 failed to show expression of the PAT enzyme. It could not be proved that any of the plants that were analysed were transgenic, rather that they had regenerated from clusters of cells or meristemoids containing only a few transformed cells and were therefore chimeric transformants.

5.5.2 UCT study

The results of the UCT study are summarised in Table 13. Of the three putative transgenic events, T1, T2 and T3, that survived selection and grew to a height of 5 cm, only T1 could be propagated in the presence of 7.5 µM PPT. The 37 clones obtained were subsequently also propagated on selection medium and transferred to 20 µM PPT when they were 5 cm tall with no detrimental effect. Furthermore, the 23 clones that were analysed in the greenhouse could all be propagated from a single leaf of each plant reintroduced to tissue culture in the presence of 7.5 µM PPT.

PCR results of T1, T2 and T3 showed a strong band only for T1. Subsequent analyses of five clones of T1 consistently confirmed that they contained the *pat* gene. ELISA results verified the transgenic nature of T1 and nine of its clones although the variation in the amounts of PAT expressed was unexpected. However, it correlated with the efficiency of regeneration from leaf explants of the respective plants cultured on 7.5 µM PPT as illustrated in Figure 12b. In this regard, C2 regenerated most efficiently, followed by C10, C20, C23, C7, C16, C14, C13 and C6.

5.5.3 Southern blot analyses

Ornithogalum is a bulbous plant with succulent leaves, which, upon wounding, produce mucous rich in arabinoglucuronomannoglycans (Burger, 1990). It was difficult to isolate DNA or RNA in this study because these polysaccharides formed a complex with the nucleic acids in solution. As a result the DNA and RNA solutions obtained in this study were generally gelatinous and dilute, which led to partial digests with restriction enzymes and inefficient probe binding, as discussed in more detail below.

Table 13 Summary of the molecular analyses of T1 and 17 of its clones; (**+**) indicates a positive result, (-) a negative result and (**nd**) that the plant was not analysed.

Line	Propagation (7.5 µM PPT)	PCR	Southern	Northern (*pat*)	Northern (*ubi*)	ELISA	Leaf paint
NT	-	-	-	-	+	-	-
T1	+	+	nd	nd	nd	+	nd
C1	+	nd	+	nd	nd	nd	nd
C2	+	nd	+	+	+	+	+
C6	+	+	+	+	+	+	+
C7	+	+	+	+	+	+	+
C9	+	nd	nd	nd	nd	nd	nd
C10	+	nd	nd	nd	nd	+	nd
C12	+	+	nd	+	+	nd	+
C13	+	+	+	+	+	+	+
C14	+	+	+	+	+	+	+
C16	+	nd	nd	+	+	+	nd
C17	+	nd	nd	nd	nd	nd	+
C18	+	nd	nd	nd	nd	nd	+
C19	+	nd	nd	nd	nd	nd	+
C20	+	nd	+	nd	nd	+	+
C21	+	nd	nd	nd	nd	nd	+
C22	+	nd	+	nd	nd	nd	+
C23	+	nd	nd	nd	nd	+	+

DNA extractions. DNA isolation from leaves was optimised with plants cultured *in vitro*. Good quality DNA was obtained with the method of Richards (1989) which included CsCl gradient purification. Yields varied between 10 and 20 µg/g leaf tissue and digested efficiently with both *EcoR*I and *Bgl*II. However, polysaccharides complicated extractions from leaves from greenhouse plants. DNA agglomerated and degraded in CsCl gradients and the EtBr could not be removed efficiently. These polysaccharides could not be eliminated by differential precipitation with NaCl and ethanol (Fang *et al.*, 1992; Michaels *et al.*, 1994). Burger (1990) reported that hemicellulase (Sigma) degraded these polysaccharides in extractions of ornithogalum mosaic virus from *Ornithogalum* leaves. However, addition of hemicellulase to homogenised leaves prior to DNA isolation in this study, caused DNA degradation by DNase I. This was unavoidable since the pH 5.2 optimum of hemicellulase prevented inclusion of DNAse inhibitors such as EDTA and no DNAse- or RNase-free hemicellulase was available.

Subsequently, various protocols were compared for DNA isolation from leaves of greenhouse plants. The method of Dellaporta *et al.* (1983) was used as a basis, in combination with CsCl gradient purification and an increased extraction buffer volume. CTAB extraction buffers (Rogers and Bendich, 1994) and DNA precipitations at room temperature instead of –20 °C were also tested. In addition, various concentrations of NaCl and ethanol were used for differential precipitation as Fang *et al.* (1992) reported that 0.25 M NaCl and 0.35 volumes EtOH differentially precipitates polysaccharides from a DNA solution and Michaels *et al.* (1994) found that 1.5 to 2.5 M NaCl and two volumes of ethanol precipitated DNA but not polysaccharides. However, for *Ornithogalum*, the polysaccharide-DNA complex always co-precipitated.

The best DNA yields were obtained with the method of Dellaporta *et al.* (1983). This DNA often digested poorly but were improved by the addition of two phenol:chloroform (1:1) purification steps but the DNA solutions remained viscous and the yields (15 to 40 µg/g fresh tissue) and concentrations (0.08 to 0.2 µg/µl) compared poorly to that obtained by Dellaporta of 50 - 100 µg/g fresh tissue (1 µg/µl)

for tobacco, tomato, soybean, amaranthus and petunia. Yields improved when plants cultured *in vitro* or older senescing greenhouse plants were used or when plants were cultured in the dark for four to seven days prior to DNA isolation.

The viscous DNA solutions obtained often cut poorly with restriction enzymes which was evident from poor resolution on agarose gels (results not shown). Frequent mixing of the DNA-enzyme reactions, improved digestion as is evident from the even smears on the agarose gel illustrated in Figure 18a (lanes 5 to 8 and 10 to 12). Another problem that was encountered was that DNA samples that were precipitated after digestion did not dissolve in smaller volumes and the dilute samples had to be accommodated on thick gels of up to 10 mm with large wells of 3 mm x 7 mm.

Hybridisation. When the sensitivity of probe hybridisation was determined for Southern analyses, no signal was visible for the equivalent of two copies in Figure 17, lanes 4 and 13, probably because the particular dilution of the plasmid was inaccurate. The four lanes containing only plasmid DNA (lanes 7 to 10) unexpectedly showed a signal only for 10 copies of the *pat* gene. This is ascribed to diffusion of the minute amounts of plasmid from the gel during electrophoresis and transfer. To ensure that plasmid controls would be visible in all Southern blots, the equivalent of five copies of the target gene was loaded as control in all experiments with transgenic genomic DNA.

Uncut genomic DNA. Results of the Southern blot of uncut genomic DNA presented in Figure 18b (lanes 1 to 4) show that the three T1 clones tested are transgenic and that at least five copies of the *pat* gene integrated into the genomic DNA, which resolved at a molecular weight of approximately 15 kb. Signals of smaller sizes would have indicated that the gene was either not integrated or was carried on extra-chromosomal DNA elements.

EcoRi digests. *EcoR*I leberates a 1.3-kb fragment from p35SAC containing the CaMV35S promoter, *pat* gene and CaMV35S terminator (Appendix 1). Since

p35SAC was used for transformation, DNA from transgenic plants digested with *EcoR*I, would be expected to exhibit a similar 1.3-kb band containing all the copies of the *pat* gene that were inserted into the genome. In the Southern blots with genomic DNA from PPT-resistant plants, the signal for the *pat* gene in the lanes containing only p35SAC DNA digested with *EcoR*I (Figure 18b, lane 9 and Figure 19, lane 4) appears as expected at 1.3 kb according to the molecular weight markers. However, the predominant signals in the genomic DNA spiked with the *pat* gene, are retarded, indicating that electrophoresis of the genomic DNA or, more likely, the polysaccharides complexed to the genomic DNA in *Ornithogalum*, causes a shift in the perceived molecular weights of resolved fragments. The smears of larger fragment sizes also present in the lanes where the probe hybridised with the *pat* gene are probably due to partial digests, caused by insufficient access of enzyme to the DNA because of the presence of polysaccharides. Bands and smears smaller than 1.3 kb were probably due to rearrangements of the inserted DNA and degradation, respectively.

*Bgl*II *digests*. *Bgl*II cuts once inside the *pat* gene in p35SAC, linearising the plasmid (Appendix 1). It was therefore expected that for each insertion event, the *pat* gene would hybridise to two genomic DNA fragments. Tandem insertions at one locus in the genome would yield an additional band of the same size as the plasmid (3.99 kb), which will vary in intensity according to the number of copies that inserted in tandem. The latter scenario was found to be the case as illustrated in Figure 18b (lanes 12 and 13) and Figure 19 (lanes 6 to 9).

It is important to note that equal amounts of the same DNA extract were loaded for both undigested- and digested DNA on one gel as illustrated in Figure 18a lanes 2, 4 and 11 for C6 and lanes 3, 5 and 12 for C7. However, hybridisation signals in the three lanes representing each plant (Figure 18b) were not of equal strength. This indicated that either polysaccharides interfered with probe binding or that the concentration of DNA in the relatively small sector of the gel prevented hybridisation to every copy of the homologous gene. Another unexpected observation was that the Southern profiles of the different clones of T1 were not identical as expected.

However, careful observation showed that the same banding patterns did occur for all clones, although the intensities of the hybridisation signals differed. These differences in profiles are also ascribed to polysaccharides that interfered with DNA digestions and probe binding rather than differences in the DNA integration patterns in the various clones.

5.5.4 Northern blot analyses

Isolation of RNA posed similar problems as that of DNA because of the presence of polysaccharides that made extracts dilute and viscous. The single-step GTC, ß-mercaptoethanol, acid phenol protocol of Colonna-Romano *et al.* (1998) yielded most RNA but extreme care was necessary to remove the viscous aqueous phase from the cell debris. RNA did not dissolve completely in either GTC or H_2O but the remaining pellets were mostly polysaccharides. Leaves from greenhouse plants were found to contain more polysaccharides and RNA from this source could not be resolved under native or denaturing conditions. Eventually only RNA from tissue culture plants was used in northern blots.

Results from leaf paints revealed an unexpected difference in resistance to PPT amongst T1 clones and it was important to determine if was due to gene silencing, different amounts of RNA transcribed from the transgene in individual clones or different rates of transcription. To this end, the ubiquitin transcript was used as an internal standard since it is a highly conserved, constitutively expressed protein found in all eukaryotes (Garbarino *et al.*, 1995). Hybridisation with a *ubi* gene probe from *Antirrhinum majus* verified that equal amounts of RNA were loaded in different lanes of the gel presented in Figure 21b. Therefore differences observed in signal intensities with the *pat* gene in Figure 21a were due to differential expression of the gene. Figure 21a showed that C12, C14 and C16 (lanes 5, 7 and 8) expressed more PAT-RNA than C2, C6, C7 and C13 (lanes 2, 3, 4 and 6, respectively).

The *pat* probe was subsequently stripped from the membrane and probed with the *ubi* gene. The process was efficient as no signal could be detected with a Geiger counter after stripping and background signals were negligible during subsequent

detection. Hybridisation with *ubi* showed that less RNA was loaded only for C13, indicating that the different intensities of signals observed for the *pat* gene in the other plants was not caused by different amounts of RNA on the gel. However, these results did not correlate with that of the leaf paints and ELISA. Clones C7 and C12 (Table 12 and Figure 23) showed no damage in leaf paint assays compared to C6, C13 and C14 (Table 12 and Figure 24) and C2 (not tested with leaf paints) and C7 expressed the most PAT in ELISA analyses (Table 10). These results indicate that the amount of RNA transcribed does not necessarily correlate with the amount of protein expressed or herbicide tolerance, although the latter two do correlate.

The size of the *pat* transcript observed in Figure 21a was larger than expected (1.5 kb instead of 600 bp). This may have been be due to a leaky terminator, which did not terminate transcription efficiently after 600 bases but allowed the RNA polymerase to proceed past the terminator sequence on the DNA strand. This was not the case with the ubiquitin transcript, where the *ubi* probe hybridised to the expected 1.8 kb transcript (Figure 21b).

5.5.5 Greenhouse trial

Ornithogalum spp. are hardy, tolerant of senescence and acclimatise easily from tissue culture to greenhouse conditions (D. Wilson; Hadeco, personal communication). This was also found to be the case in this study. The aims of the greenhouse trials with plants from both the USDA and UCT were to determine herbicide tolerance and plant and flower morphology studies. A total of 110 plants from ten lines from the USDA study were analysed and all appeared normal (results not shown). As expected, no somaclonal variation was observed since these were clones from plants that also appeared normal in tissue culture. However, no conclusive results emerged with regard to herbicide resistance and these plants were presumed to be chimeras.

From the UCT study, 23 T1 clones and 11 non-transformed plants were transferred to the glasshouse. There were no visible differences in leaf and flower morphology between non-transformed and transgenic plants (Figure 22a). Flowers were not

market quality since it was the first season after *in vivo* culture and such bulbs have to be cultured for at least one season before they are suitable for commercial flower production (D. Wilson, personal communication).

Ignite® leaf-paint assays showed differential expression of herbicide-resistance in transgenic plants (Table 12 and Figure 24) which did not correlate with the results from northern analyses. This differential resistance could be ascribed to one of two phenomena - gene silencing or chimerism. With gene silencing the *pat* gene would have been present in Southern blots, but absent or present in low concentrations on the RNA and protein levels, correlating with low resistance. In the case of chimerism, the various clones could have regenerated from chimeric parts of T1, if T1 had originated from a cluster of cells that contained more than one transformation event, resulting in a transgenic plant containing different transgenic sectors. Such a plant could give rise to "clones" that are not identical, depending on the part of T1 they regenerated from. Such different clones would exhibit different Southern blot profiles. In both cases it would be expected that the RNA concentration of each clone should correlate with the amount of PAT protein synthesised and also herbicide resistance. Neither of these scenarios was completely satisfied in this study and the discrepancy seems to lie at the level of translation.

It is also important to realise that the levels of herbicide tolerance observed in plants cultured in the greenhouse – which were analysed in leaf paints, ELISA and regeneration efficiency on selection medium – could not be compared with the levels of transcription found in plants cultured *in vitro* and which were analysed in northern blots. The former plants had been without selection pressure for more than six months, which might have led to gene silencing while plants cultured *in vitro* in the presence of PPT were under constant selection pressure. For a clear comparison, RNA and protein should be isolated from the same leaves that are painted with herbicide to determine if there is a correlation between the amount of RNA transcribed, the amount of PAT expressed and the level of herbicide resistance.

Chapter 6

Concluding remarks

In this study, *Ornithogalum* was successfully transformed with the *pat* herbicide resistance gene using a Biorad PDS 1000/He device. Despite the relative inefficiency of this transformation system it opens the way to use gene transfer to improve *Ornithogalum* hybrids of commercial potential. Potential traits are disease resistance to ornithogalum mosaic virus (Burger and von Wechmar, 1988) and bacterial pathogens such as *Erwinia* (A Barnhoorn, Hadeco, pers. comm.), enhancing existing colours or to creation of new colours (Elomaa *et al.*, 1993) in this crop. There also exists potential to improve the fragrance of flowers by introducing novel scent genes (Dudareva *et al.*, 1996).

The intended comparison between traditional biolistics and the Taxi could not be made in this study. Only one stable transformation event was obtained with the Taxi protocol and is insufficient for such a comparison. A more efficient transformation protocol needs to be developed to obtain many transformation events, both from traditional biolistics and from the Taxi. Such plants will then have to be analysed with Southern and northern blots to determine if the Taxi is more efficient for directing low copy number insertions with concomitant stable expression of the transgenic trait in *Ornithogalum*. This is the logical next step but will take at least another two years. Suggestions to improve the current system are discussed in more detail.

A large number of variables were involved in the various aspects of this study ranging from molecular biology, tissue culture, gene transfer, selection of and regeneration from transgenic tissues. It was therefore difficult to test a large range of variables and to establish the best possible method. A balance had to be found between testing as many variables as practically possible in a methodical way.

On the molecular biology level it is important to choose the best vector construct for the goal of the project. More promoters should be evaluated in transient expression assays in future, preferably all contained in the same background, which may require

subcloning each respective promoter in front of the same reporter gene on one vector backbone. Once the most efficient promoter has been identified, the same strategy has to be applied to obtain the target transgene in the best conformation with the smallest amount of undesirable DNA, including bacterial selectable marker genes.

The tissue culture protocol developed in this study provided sufficient regenerable target tissue but the transformation efficiency and subsequent regeneration of transgenic plants on selection medium was very low. Attention will be paid to find more suitable target tissues for bombardment or to improve the incidence of stable transformation events in the current system. As was illustrated in the histological study, there are a number of pathways by which *Ornithogalum* can be regenerated *in vitro* from both differentiated as well as undifferentiated explant tissues. It should be possible to exploit some of these pathways in transformation studies to improve the efficiency of the protocol described in this study. For example, preliminary observations have shown that somatic embryos can be induced on leaf explants, therefore future attempts will focus on using thin cell layers from the leaf, where the explant consists of as small a number of identifiable cell types as possible. Such a system may allow better identification of the target cells involved in differentiation.

Another aspect of the transformation protocol that warrants further investigation is avoiding regeneration of escapes and chimeras. To this end, bombarded callus should be cultured for a longer period on callus proliferation medium supplemented with a sub-lethal concentration of selection agent to enable non-transformed tissues to survive but not proliferate, while transformed cells or clusters of transformed cells will proliferate. Subsequent transfer of callus tissue to regeneration medium containing a lethal concentration of selection agent, should ensure that only clusters of transformed cells will differentiate into meristemoids and subsequently plants of which a high percentage will be stably transformed.

Identification of chimeras as well as the frequency at which they occur in a specific transformation protocol can be simplified by bombarding tissues with an efficient promoter driving a *bar*GUS fusion. Such experiments should generate GUS-expressing plants that can survive on selection medium and subsequent staining

with X-Gluc will show up chimeras, which will give an indication of the incidence of stable transformation in relation to transient expression of the reporter gene.

Mutation studies with monocot plants have shown that during selection of solid mutants through regeneration from leaves of plants submitted to mutagens, adventitious shoots are almost certain to arise from single cells (Broertjies and van Harten, 1988). Therefore, regeneration of chimeras from *Ornithogalum* can probably be further reduced by propagation of a second generation of plants from leaf explants of putative transgenic plants on selection medium.

Another aspect, which should receive more attention, is the improvement of nucleic acid isolation protocols. Elimination of polysaccharides from DNA solutions will enable estimation of the exact number of copies and integration events that occur in individual transgenic plants. Furthermore, an efficient RNA extraction protocol will enable isolation of RNA from greenhouse grown plants so that direct comparisons can be made between the amount of RNA transcribed and the levels of transgene expression and resistance observed. This information will provide a clearer picture of the incidence of transgene silencing in transgenic *Ornithogalum* plants.

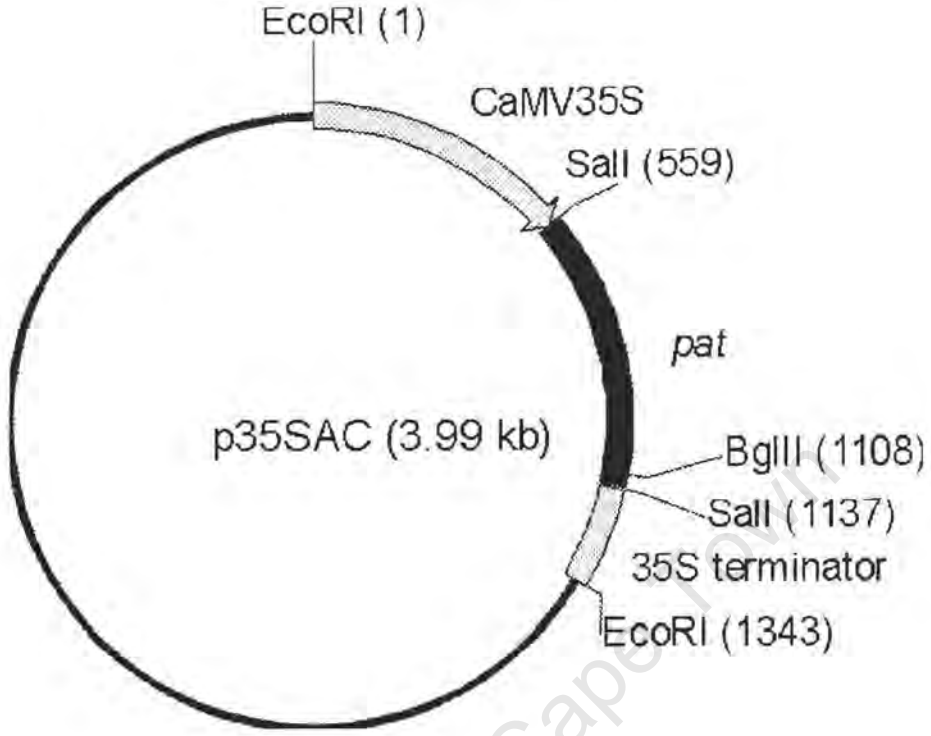
Agricultural biotechnology has entered a new era where it is no longer sufficient merely to introduce a gene into a plant. Transgenes should be introduced into the economically important lines of crops in single copy and without the integration of unnecessary vector "backbone" sequences. Expression of transgenes must be predictable and consistent among numerous independent transformants. Recent research has more clearly defined these problems and pointed the way to their solution (Gelvin, 1998). In this regard, a method was reported that eliminates selectable marker genes from transgenic plants. Ebinuma *et al.* (1997) included the isopentenyl transferase gene (*ipt*), which confers a "shooty" phenotype to transgenic plants, on a transposable element. Elimination of the transposon after regeneration eliminated the marker gene and restored the normal phenotype to transgenic plants. In another case, Joersebo *et al.*, (1998) used mannose selection instead of undesirable antibiotic resistance selectable marker genes.

Beyond the optimisation of transformation protocols for individual crops, there are two obstacles that hinder the commercialisation of many transgenic products. One is legal, the various patents pertaining to specific aspects of transformation technology, and the other is public perception. The former is aimed at protecting investors' interests to ensure returns in the long run. However, licensing agreements to use patented technology is often cumbersome, expensive and difficult to obtain.

A growing percentage of the public shows concern for the environment and a safe supply of residue-free food. In most industrialised countries these concerns now extend far beyond environmental activists, and their proponents is shaping up as a political force to be reckoned with. Some of the concern is directed toward what is perceived as an indiscriminate and excessive use of agrochemicals to control plant pests, weeds and diseases and low-input, sustainable agriculture is seen as a necessary and viable alternative. By their very nature genetically resistant crops fit this concept perfectly. Curiously, it is the same societal groups that are emotionally and fundamentally at arms with genetically engineered organisms, which they reject as scientists tinkering with creation. The public's desire for farming practices that minimise adverse effects on the environment may become a decisive factor in the debate in favour of genetically engineered crops but acceptance of biotechnological products, including genetically engineered crop plants, is still an unresolved issue. The debate is likely to continue for several years and is badly in need of facts and rationale. Only time will tell if a majority of consumers and farmers will come to accept the benefits of genetically engineered crops over their perceived risks. In this regard, ornamental plants can provide an excellent "minimal risk, minimal anxiety" product to serve as example for educating the general public about the advantages of gene transfer to plants.

Appendix 1

Plasmid map



Plasmid map of p35SAC, used for stable transformation studies.

Appendix 2

Media, buffers, solutions and general techniques

A2.1 Tissue culture media

A2.1.1 Plant growth regulator stock solutions

Crystallised growth regulators were dissolved in a few drops of EtOH or 0.1N NaOH (as indicated), then diluted to a final concentration of 0.5 mg/ml in dH_2O with pH set at 5.6 to 5.8.

- BA 1N NaOH
- Biotin H_2O
- 2,4-D $EtOH/H_2O$
- Dicamba $EtOH/H_2O$
- Folic acid 1N NaOH
- IAA EtOH/1N NaOH
- Kinetin 1N NaOH
- NAA 1N NaOH
- PPT Dilute in H_2O

A2.1.2 MS basal medium for maintenance and rooting of plants

4.81 g MS salts and vitamins
30 g sugar
Set pH at 5.8
Make up to 1 l with dH_2O
Add 8 g agar and autoclave

A2.1.3 Media for regeneration from leaf explants

- MSB
MS basal medium
BA 0.5 mg
Make up to 1 l with dH_2O
Set pH at 5.8
Add 8 g agar and autoclave

- MSB2
MS basal medium
BA 2.0 mg
Make up to 1 l with dH_2O
Set pH at 5.8
Add 8 g agar and autoclave

- MSN
MS basal medium
NAA 0.1 mg
Make up to 1 l with dH2O
Set pH at 5.8
Add 8 g agar and autoclave

- MSNB
MS basal medium
BA 0.5 mg
NAA 0.1 mg
Make up to 1 l with dH2O
Set pH at 5.8
Add 8 g agar and autoclave

- MSNB2
MS basal medium
BA 2.0 mg
NAA 0.1 mg
Make up to 1 l with dH2O
Set pH at 5.8
Add 8 g agar and autoclave

A2.1.4 Callus initiation media

- K2

MS basal medium
2,4-D 1 mg
Kinetin 0.2 mg
Folic acid 0.5 mg
Biotin 0.05 mg
Set pH at 5.8
Make up to 1 l with dH2O
Add 8 g agar and autoclave

- K2D

MS basal medium
Dicamba 1 mg
Kinetin 0.2 mg
Folic acid 0.5 mg
Biotin 0.05 mg
Set pH at 5.8
Make up to 1 l with dH2O
Add 8 g agar and autoclave

- K2N

MS basal medium
NAA 5 mg
Kinetin 0.2 mg
Folic acid 0.5 mg
Biotin 0.05 mg
Set pH at 5.8
Make up to 1 l with dH2O
Add 8 g agar and autoclave

- K2I

MS basal medium
IAA 8 mg
Kinetin 0.2 mg
Folic acid 0.5 mg
Biotin 0.05 mg
Set pH at 5.8
Make up to 1 l with dH2O
Add 8 g agar and autoclave

A2.1.5 Media for regeneration from callus initiated on:

- K2N

MS basal medium
BA 1 mg
Kinetin 0.2 mg
Set pH at 5.8
Make up to 1 l with dH2O
Add 8 g agar and autoclave

- K2D

MS basal medium
BA 1 mg
Kinetin 0.2 mg
Set pH at 5.8
Make up to 1 l with dH2O
Add 8 g agar and autoclave

A2.2 Plasmid isolation and manipulation of nucleic acids

A2.2.1 Luria Bertani (LB) broth

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	5 g

Make up to 1 l with dH2O and autoclave
Solid media contained 1.2% (w/v) agar

A2.2.2 Antibiotics

Ampicillin stock solution (100 mg/ml in dH_2O) was added to media cooled to 50 °C and were stored for no longer than two weeks at 4 °C.

A2.2.3 Denhard's solution (10x) (Sambrook *et al.*, 1989)

Ficoll	1 g
Polyvinylpirrolidone	1 g
BSA (fraction V)	1 g

Make up to 100 ml with dH_2O, filter sterilise and store at –20 °C.

A2.2.4 EDTA (0.5 M, pH 8.0) (Sambrook *et al.*, 1989)

EDTA.$2H_2O$	168.1 g
dH_2O	to 1000 ml

EDTA will only dissolve when the pH has been adjusted to 8.0 (Use approximately 20 g of NaOH pellets for this purpose)

A2.2.5 Ethidium bromide solution

(2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide)
A solution of 10 mg/ml was made with dH_2O and stored in a dark bottle.

A2.2.6 Isopropanol (salt saturated)

Isopropanol was saturated with aqueous 5 M NaCl, 10 mM Tris, 1 mM EDTA, (pH 8.0)

A2.2.7 TAE buffer (50x)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 *M* stock, pH 8.0)	100 ml

A2.3 Microprojectile bombardment

A2.3.1 Solutions for DNA precipitation

- Sterile gold microprojectiles
Weigh out 60 mg of gold microprojectiles (Biorad)
Add 1 ml 70% EtOH and vortex for 15 min
Wash 3 times with sterile dH_2O by repeated vortex and centrifugation
Resuspend in 1 ml 50% (v/v) sterile glycerol
Aliquot 50 µl quantities into sterile eppendorf tubes and store at –20 °C

- $CaCl_2$ (2.5 M)
3.68 g of $CaCl_2$ (MW = 147.02)
Make up to 10 ml with dH_2O
Filter sterilise, aliquot into sterile eppendorf tubes and store at -20 °C

- Spermidine (100 mM)
Prepare a 10M stock solution by adding 689 µl sterile dH_2O to 1g spermidine, free base (Sigma)
Add 10 µl stock to 990 µl sterile dH_2O in 10 eppendorf tubes.
Store at -20 °C, thaw one tube at a time before use and discard the solution that remains after precipitations done on a single day.
Store the 10M stock solution at -20 °C and thaw only twice for subsequent dilution to 100 mM.

A2.3.2 Manipulation of bombarded callus

- X-Gluc stain

X-Gluc (1mM)	50 mg
NaH_2PO_4 (200 mM, pH 7)	50 ml
Na_2EDTA (0.5 M)	0.5 ml
Sarcocyl (10%)	0.5 ml

Make up to 100 ml with dH_2O, filter sterilise and store in the dark at 4°C. If the stain discolour to blue, discard it

- PPT stock solution (25 mg/ml)
Add 2.5 ml Ignite® herbicide to 17.5 ml dH_2O and mix well
Filter sterilise and aliquot into sterile eppendorf tubes
Store at –20 °C until needed and store the remaining solution at 4 °C.

A2.4 General techniques

A2.4.1 Plasmid preparation (large scale)

A 200 ml culture of *E.coli* containing the plasmid to be isolated were grown overnight at 37 °C in the presence of the appropriate antibiotic. Cells were harvested by centrifugation at 6 000 x g and resuspended in 4 ml Solution I (50 mM glucose, 25 mM Tris, pH 8.0). After 5 min at RT, 8 ml Solution II (0.2 M NaOH, 1% (w/v) SDS) was added, the mixture was kept on ice for 5 min, followed by the addition of 6 ml cold Solution III (5 M KOAc, pH 4.8). The sample was vortexed briefly, left on ice for 5 min and the cell debris sedimented by centrifugation at 12 000 x g for 10 min. An equal volume of isopropanol was added to the supernatant and the DNA was precipitated by centrifugation at 27 000 x g for 15 min. The DNA pellet was washed with 70 % EtOH, resuspended in 4.2 ml TE buffer and purified by isopicnic CsCl-EtBr ultracentrifugation (Sambrook *et al.*, 1989).

The plasmid DNA solution was prepared for ultracentrifugation by addition of CsCl (1 mg/ml) and EtBr (0.5 ml of a 10 mg/ml stock), followed by a centrifugation at 27 000 x g for 10 min to precipitate all debris. The refractive index of the supernatant was adjusted to 1.396, the sample sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged for 16 h at 60 000 rpm or 4 h at 90 000 rpm at 15 °C in a Beckman NVT 90 rotor. The plasmid DNA band was visualised by long wave UV light (350 nm) and removed in the smallest possible volume. The EtBr was removed by extraction (at least three times) with NaCl-saturated isopropanol. The DNA was precipitated from the CsCl solution by the addition of two volumes of H_2O, followed by addition of an equal volume of isopropanol and centrifugation at 13 000 x g for 15 min. The pellet was resuspended in 200 µl TE buffer and the DNA concentration (1:1000 dilution in TNE buffer (10 mM Tris, pH 7.4, 0.1 M NaCl, 10 mM EDTA)) was determined with a fluorometer (Sequoia-Turner).

A2.4.2 Determination of protein concentrations

Protein concentrations in solutions were determined bu the method of Bradford (1976). Assays were performed in triplicate using disposable cuvettes. Reactions contained protein solution (0.8 ml) and 200 µl Bradford solution (Biorad). The mixture was left to stand for 5 min at room temperature and the optical density of the reaction measured at 595 nm. Protein concentrations were calculated using a standard curve (BSA fraction V; 10 – 100 µg/ml). Protein samples were diluted so that the optical densities did not exceed 0.8.

A2.4.3 Restriction enzyme digests of:

Plasmid DNA

Combine: 1 µg plasmid DNA
1 µl of 10 x reaction buffer
Make up to 10 µl with sterile dH_2O and mix well
Add 0.1 µl restriction enzyme (10 u/µl)
Incubate for 1 h at 37°C unless stated otherwise

Plant genomic DNA

Combine: 20 µg genomic DNA
20 µl of 10 x reaction buffer
(buffer H for *EcoR*i and M for *Bgl*ii)
Make up to 200 µl with sterile dH_2O and mix well
Add 2 µl restriction enzyme (10 u/µl)
Incubate for 24 h at 37°C
Mix by pipetting every 2 h

A2.4.4 Bombardment with the Taxi

Denaturation of plasmid DNA

Combine: 1 µg linearised plasmid DNA
7.5 µl TE buffer
Make up to 75 µl with dH_2O
Boil for 10 min and immediately transfer to ice.

Preparation of DNA-histone H1 complex

Dissolve 10 µg of lyophilised histone H1 in 1 ml sterile dH_2O
Aliquot 1.8 µl volumes and store at –70 °C until needed.
Combine: 1.8 µl (18 µg histone H1)
2.5 µl TE buffer
20.7 µl sterile dH_2O
Mix the histone solution with the denatured DNA solution by vortexing for a few seconds. Incubate on ice for 30 min.

A2.4.5 Capillary transfer of nucleic acids

- After electrophoresis, stain the agarose gel for 30 min with EtBr (1µg/ml) in dH_2O and take a picture on a trans-illuminator.
- Rinse the gel with dH_2O.
- Fragment the DNA by immersion for 15 min in 0.25 N HCl (25 ml 10 M HCl in 1 l dH_2O) with gentle shaking.
- Denature the double-stranded DNA by soaking the gel for 30 min in

NaCl (5 M)	200 ml
NaOH (10 M)	50 ml
Make up to 1 l with dH_2O	

- Neutralise the gel by soaking for 30 min in

Tris-HCl, (1 M, pH 8.0)	500 ml
NaCl (5 M)	100 ml
Make up to 1 l with dH_2O	

- Float the nylon membrane (Magna charge) on dH_2O, then immerse until it is thoroughly wet.
- Use a transfer buffer of 20 x SSC

NaCl (5 M)	600 ml
Na Citrate (1 M, pH 7.0)	300 ml
Make up to 1 l with dH_2O	

- Cut 3 pieces of 3MM filter paper 10 cm longer than the glass plate to be used as support for the capillary transfer.
- Saturate the filter paper with the transfer buffer and place it on top of the glass plate.
- Place the gel on top of the filter paper and the membrane (exact same size as the gel) on top of the gel.
- Place 3 pieces of filter paper cut to the size of the gel over the assembly. Throughout the assembly, do not allow the paper on top of the gel to contact the paper below the gel by placing strips of parafilm or X-ray film around the sides of the gel.
- Place the glass plate and the gel assembly on top of a glass baking tray. Allow the bottom layers of filter paper to overhang into the transfer buffer in the glass baking tray.
- Place a 5 cm stack of paper towels on top of the gel assembly and secure it with a light weight. Make sure that the filter paper under the gel is completely saturated. The wicking action of the solution through the gel and up the paper towels allows the solution to transfer the DNA or RNA molecules to the membrane.
- Leave the assembly in a cool place for 3 h to overnight. If the paper towels become saturated with transfer buffer, replace them with dry ones.
- After transfer, stain the gel with 0.1 µg/ml EtBr to check the transfer efficiency.

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