Development of an *Agrobacterium* transformation system for onion (*Allium cepa* L.)

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Abstract

Onion (*Allium cepa*) bulbs of the New Zealand-bred cultivars 'Pukekohe Longkeeper' and 'Early Longkeeper' produced tumourous growths after inoculation with 25 virulent strains of *Agrobacterium tumefaciens*, *A. rubi* and *A. rhizogenes*. The majority of these tumours produced nopaline, indicating that tumour cells were transformed. Some excised tumours produced roots in sterile culture.

Eight onion genotypes were screened in tissue culture for callus formation, regeneration of plantlets from callus and clonal multiplication by shoot proliferation. All genotypes could be clonally multiplied and four were readily regenerable from callus. A technique for plantlet multiplication, which uses longitudinally-bisected stems of *in vitro*-germinated onion seedlings as explants, was developed. Onion ('Pukekohe Longkeeper', 'Southport White Globe', 'Japanese Saporo Yellow' and 'Hikeeper F1') protoplasts were isolated and cultured on a range of media. These protoplasts formed new cell walls and sometimes divided, but only first divisions were regularly seen.

Kanamycin, geneticin (G418), hygromycin and chlorsulfuron were evaluated for their use as selective agents in onion transformation experiments. Tissues surveyed for sensitivity to these selective agents included seeds and seedlings on germination and callusing media, established callus on callusing and regeneration media, and shoot cultures on shoot proliferation medium. Hygromycin was shown to be the antibiotic most toxic to tissues of all the surveyed onion cultivars, with effects being obvious in all tissues after 4-5 weeks of culture on concentrations as low as 20 mgl^{-1}. Kanamycin was shown to be the least toxic of the selection agents surveyed. The kanamycin analogue G418 was considerably more toxic to most onion cultures than kanamycin. However, responses of cultures to G418 were slower than those to hygromycin. The herbicide chlorsulfuron was also shown to be toxic to onion seedlings and shoot cultures.

The ability of *Agrobacterium tumefaciens* to transfer foreign genes to *A. cepa* was

demonstrated. A single, putatively transformed plantlet (RC1), was regenerated from an onion seedling stem via callus, following co-cultivation of stem explants with *Agrobacterium* strain LBA4404 harbouring the binary vector pKIWI110. In addition, 41 axillary or adventitious shoots which grew directly from basal plates injected *in vitro* with four strains of *A. tumefaciens* (each harbouring the binary vectors pKIWI110 or pGA643) exhibited resistance to G418 in culture. The binary vectors used carry the neomycin phosphotransferase II gene (nptII) controlled by the nopaline synthase (*nos*) promoter. Both RC1 and some of the shoots growing from basal plate explants produced roots when grown on culture media supplemented with G418. Southern analyses showed that fragments of DNA from RC1 and from five of the 41 G418-resistant shoots hybridized to a 1.25 kbp nptII probe. β-glucuronidase (GUS) activity was detected in over half of the plantlets derived from basal plate tissue injected with *A. tumefaciens* strains LBA4404 or C58, both of which harboured pKIWI110. Molecular and phenotypic evidence suggested that the putatively transformed plants produced from injected basal plate tissues were chimeric.

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Abbreviations

APH	aminoglycoside phosphotransferase type II (apt (3')II)
BA	6-benzylaminopurine
BDS	B5 plant tissue culture medium as modified by Dunstan and Short (1977)
bp	base pairs
B5	Gamborg (1968) plant tissue culture medium
CAT	chloramphenicol acetyltransferase
CaMV 35S	35S promoter of cauliflower mosaic virus
cm	centimetre
CTAB	cetyltrimethylammonium bromide
cv	cultivar
2,4-D	2,4-dichlorophenoxyacetic acid
DGT	direct gene transfer
DNA	deoxyribonucleic acid
μE	micro Einsteins
'ELK'	'Early Longkeeper'
GUS	β-glucuronidase
G418	geneticin
h	hour/s
2iP	6-(3-methyl-2-buten-1-ylamino)-purine
'JSY'	'Japanese Saporo Yellow'
kbp	kilobase pairs
KM 8p	protoplast culture medium devised by Kao and Michayluk (1975)
LB	Luria Broth for bacterial culture (Miller, 1972)
LSD	least significant difference
min	minute/s
MS	Murashige and Skoog (1962) basal plant tissue culture medium
NAA	α-naphthalene acetic acid
nos	nopaline synthase promoter from *Agrobacterium*
NPTII	neomycin phosphotransferase II
ns	not significant
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGR	plant growth regulator
picloram	4-amino-3,5,6,-trichloropicolinic acid
'PLK'	'Pukekohe Longkeeper'
Ri	root-inducing
SDS	sodium dodecyl sulphate
sec	second/s
ss	single stranded (DNA)
'SWG'	'Southport White Globe'
T-complex	T-DNA-protein complex
T-DNA	transferred DNA

TDZ	thidiazuron (n-phenyl-n'-1,2,3-thiadiazol-5-yl urea)
Ti	tumour-inducing
V-KM	protoplast culture medium of Kao and Michayluk (1975) as modified by Boekelmann and Roest (1983)
W-MS	Murashige and Skoog (1962) medium as modified by Wang *et al.*(1986) for the culture of onion protoplasts
*	significant at the 5% level
**	significant at the 1% level
***	significant at the 0.1% level

Chapter 1: General Introduction

1.1 *Allium cepa*

The onion (*Allium cepa* L.) is an important monocotyledonous vegetable crop, which has historically been classified with other alliums into the Liliaceae family (Cronquist, 1968; Takhtajan, 1973). The *Allium* genus has also been classified into the Amaryllidaceae family by at least one prominent taxonomist (Hutchinson, 1973), and more recently, following the trend towards small, monophyletic families, it has been placed in the Alliaceae family (Dahlgren *et al.*, 1985). *A. cepa* is usually propagated from seed and is generally regarded as an outcrossing species. Individual flowers in the onion inflorescence are highly protandrous, effectively preventing the self-pollination of a single flower (Currah and Ockendon, 1978). *A. cepa* is also propagated from bulbs or small sets (bulblets). Field-grown onions are mainly cultivated as a biennial crop, having a seed-to-seed generation time of two years. This generation time of onions is long in comparison to those of many other major crops.

The onion is grown on all continents of the world, mainly for its flavouring qualities, and has become an essential part of the human diet. Worldwide, 2.6 million metric tonnes of onion edible dry material are produced annually (Harlan, 1992). It is estimated that the value of world annual production of bulb onions approaches $US 5 billion, more than 90% of which is consumed within the countries of production (Rabinowitch and Brewster, 1990). In New Zealand, the onion is grown for both local consumption and for the export market. Onions are New Zealand's fourth largest horticultural revenue earner after kiwifruit, apples and squash. New Zealand produces approximately 80,000 tonnes of onions annually, of which about half is exported, principally to Europe and Japan (Hale *et al.*, 1992). The two cultivars

most commonly exported from New Zealand are 'Pukekohe Longkeeper' and 'Early Longkeeper', both New Zealand-bred cultivars which store well. Although their keeping properties are excellent, these cultivars have been criticized, particularly by Japanese consumers, for their high pungency and lack of sweetness (McPherson *et al.*, 1992). The breeding of new characteristics into onions by conventional plant breeding methods is a time-consuming process that can take up to several generations, and so quicker ways to insert desirable genes into the onion genome are being sought.

1.2 The introduction of foreign genes into plants - an overview

Since early reports of the regeneration of transgenic plants (Horsch *et al.*, 1984; Paszkowski *et al.*, 1984), the area of gene transfer into plants has made substantial and significant progress (Gasser and Fraley, 1989). Many laboratories worldwide have transferred genes into a wide variety of plants including 'easy' plants, e.g. tobacco (*Nicotiana tabacum*) and petunia (*Petunia hybrida*), and 'difficult' or recalcitrant plants, e.g. soybean (*Glycine max*) or cereals, e.g. maize (*Zea mays*) and rice (*Oryza sativa*). Currently, *Agrobacterium*-mediated gene transfer is probably the most commonly used vehicle for transporting foreign DNA into plant cells (Grant *et al.*, 1991). This DNA can subsequently be stably integrated into the nuclear genome of a plant and transcribed and translated as normal DNA. Expression of the introduced gene or genes can usually be monitored. However, *Agrobacterium*-mediated gene transfer cannot yet be successfully used for all plant species. Susceptibility, or lack of susceptibility, of plants to *Agrobacterium* has been attributed to a number of factors, some of which will be discussed in this thesis. For those plants not susceptible to *Agrobacterium* there are several other effective ways to enable direct gene transfer (DGT) into plant cells. These range from the uptake of DNA into isolated protoplasts, which is mediated by chemical procedures or electroporation, to micro- and macroinjection, and bombardment of tissue with high-velocity particles (microprojectiles).

In this introductory chapter, the non-*Agrobacterium* methods of gene transfer into plants will be briefly reviewed. These will include the direct gene transfer techniques already mentioned, and a range of techniques which are less commonly used. *Agrobacterium*-mediated gene transfer and how it could be used for onions will then be reviewed in more detail.

1.2.1 Direct gene transfer into protoplasts

DGT into plant cells requires the removal of the plant cell wall to form protoplasts. In comparison to the transformation of bacteria, yeast and animal cells, the transformation of plant protoplasts via the introduction of 'naked' DNA has been a relatively recent development. DNA uptake into, and transformation of plant protoplasts was first demonstrated conclusively by Davey *et al.* (1980), who isolated Ti plasmid from *Agrobacterium tumefaciens* and applied it to *Petunia* protoplasts in the presence of poly-L-ornithine, or polyethylene glycol (PEG) and Ca^{2+}. The presence of Ti DNA in the plant genome was demonstrated phenotypically by hormone-independent growth and the production of opines, and also by Southern analysis of DNA extracted from transformants. Subsequent development of anti-biotic resistance markers also allowed for positive selection of transformed plant cells, and led to the development of more simplified protoplast transformation techniques, using small plasmid vectors rather than the large Ti plasmids. It was first shown that protoplasts could be transformed with small, simple plasmids by Pazskowski *et al.* (1984). They demonstrated the uptake, integration and expression of the kanamycin resistance gene aminoglycoside phosphotransferase type II (apt(3')II) in protoplasts which had been transformed with pABD1. This was done by way of Southern analysis and assays for APH(3')II enzyme activity. The pABD1 plasmid used was based on pUC8, and contained aph(3')II from the bacterial transposon Tn5 under the control of the cauliflower mosaic virus (CaMV) gene VI expression signals. CaMV expression signals were chosen because gene VI is expressed at very high levels in plant cells during viral infection (Xiong *et al.*, 1982). The presence and expression of the aph(3')II gene was retained through regeneration into plants, and was also inherited by the progeny of subsequent generations.

Since the use of the large Ti and other smaller plasmids to deliver DNA into protoplasts, the methodology of inserting DNA into protoplasts has developed considerably, with chemical and electrical methods now routinely in use. Chemical methods almost always include the use of PEG (Antonelli and Stadler, 1989; Krens *et al.*, 1982). The electrical method most commonly in use involves a technique known as electroporation, in which the application of high voltage pulses to protoplasts induces localized rearrangement of membrane components, resulting in transient membrane pores through which macromolecules such as DNA may pass. This technique was developed for plants by Fromm *et al.* (1985) and was originally used to transfer DNA to *Daucus carota* (carrot), *Nicotiana tabacum* (tobacco) and *Zea mays* (maize) cells. These cells were transiently transformed by supercoiled plasmid DNA carrying the chloramphenicol acetyltransferase (cat) gene.

Transient gene expression, i.e. expression of introduced genes that have not been integrated into the host genome, provides information about the expression of foreign genes and the activity of their promoters in plant cells. Expression is usually measured as the activity of a gene product. Measuring levels of transient gene expression in protoplasts derived from different plant organs can provide information about promoter expression in cells from these organs and in specialized types of cells. However the applicability of these assays is limited, as protoplasts are not always physiologically identical to the cells from which they are derived (Dekeyser *et al.*, 1990).

Another less commonly used method for the insertion of foreign DNA into protoplasts is that of microinjection. This involves immobilizing protoplasts, usually in an agarose matrix (Lawrence and Davies, 1985) and injecting them with a fine glass capillary needle through which DNA is passed into the cell's nucleus. This technique was first reported to be successful in plant cells by Steinbiss and Stabel (1983).

If the objective of DGT into plant cells is the recovery of transgenic plants, removing the cell wall to allow entry of DNA can create the additional problem of

regenerating plants from single protoplasts. Regeneration of plants from protoplasts remains the limitation to producing transgenics in many plant species, more commonly monocotyledonous plants and particularly some cereals, all of which belong to the Poaceae (Gramineae) family. Although regeneration from protoplasts may not be possible, the regeneration barrier need not stop DGT to such recalcitrant species, as important information about gene expression can be gathered by checking for transient expression of foreign genes within hours of inserting the DNA (Pröls *et al.*, 1988). Such evaluation of transient expression can occur without prior cell division or integration of foreign DNA into the plant cell genome.

Published protocols for the isolation of protoplasts from onion (Zeiger and Hepler, 1976; Ayeh, 1982; Tashiro *et al.*, 1984) and garlic (*Allium sativum* L.) (Opatrny and Havranek, 1977) tissue do exist, but to date there has been only one published report of plantlets being regenerated from onion protoplasts (Wang *et al.*, 1986). The original account of this is written in Chinese and is seldom cited in the literature.

1.3 Microprojectile bombardment (the Particle Gun)

As regeneration from protoplasts is still not readily achievable in some species, alternative methods of inserting DNA into plants, that bypass the protoplast-to-plant regeneration step, have since been developed. The most widely used of these is microprojectile bombardment. Following an initial demonstration of strong transient reporter gene expression in onion epidermal tissue bombarded with DNA-coated microprojectiles (Klein *et al.*, 1987), the technique was adopted and modified successfully by numerous groups worldwide. This technique employs high velocity metal particles (microprojectiles) to deliver biologically active DNA into plant cells. The original concept has been described in detail by Sanford (1988) but briefly, it involves DNA-coated microprojectiles being placed on the front surface of a macro-projectile which is then propelled by either a gunpowder charge or a gas pulse towards a stop plate with a small pore. The macroprojectile is stopped by the stop

plate, but accelerated microprojectiles continue through the pore in a vacuum chamber into the target tissue, thus delivering the DNA into the plant cells. Christou *et al.* (1988) demonstrated that the process could be used to deliver biologically active DNA into living cells and result in the recovery of stable transformants. The ability to deliver foreign DNA into regenerable cells, tissues and organs appears to provide a 'foolproof' method for achieving truly genotype-independent transformation in many agronomic crops, bypassing *Agrobacterium* host-specificity. Due to the physical nature of the technique, there is no biological limitation to the actual DNA delivery process, thus genotype is not a limiting factor. However, regeneration of plants from the 'shot' explants may be genotype-dependent. Reports of transgenic plants obtained through this method have been widespread and have included crop species as diverse as cotton (*Gossypium hirsutum* L.) (Finer and McMullen, 1990), soybean (Wang *et al.*, 1988), maize (Klein *et al.*, 1989; Gordon-Kamm *et al.*, 1990) and wheat (*Triticum aestivum*) (Vasil *et al.*, 1992).

Although the regeneration-from-single-cell step can be bypassed when using the particle gun, Potrykus (1991) considers that this technique does not necessarily offer much more hope for recalcitrant species. In a review of gene transfer methods he points out that plants difficult to transform with *Agrobacterium* probably have very few 'competent' cells, and that the particle has to reach these rare cells by a random hit, after which the DNA has to integrate into the genome of these cells. Because of the low conversion rate of transient events to stable integrative events with the particle gun system, he expects that integrative transformation in plants recalcitrant to transformation will be rare.

1.4 Other methods of direct gene transfer

A range of other non-*Agrobacterium* methods have been used to attempt direct insertion of DNA into plants on the multicellular scale. These include microinjection into zygotic and microspore-derived proembryos (Neuhaus and Spangenberg, 1990),

soaking dry seeds (Ledoux and Huart, 1974) or embryos (Senaratna *et al.*, 1991) in DNA, pollen transformation (Ohta, 1986), the 'pollen tube pathway' (Luo and Wu, 1988), electroporation of walled cells (Lindsey and Jones, 1990) or tissue slices (Dekeyser *et al.*, 1990), electrophoresis using shoot meristems (Ahokas, 1989), macroinjection (De la Pena *et al.*, 1987), liposome fusion (Caboche, 1990), liposome injection (Lucas *et al.*, 1990), microlaser (Weber *et al.*, 1988) and directly pipetting DNA into (wheat) flower spikelets (Hess *et al.*, 1990). None of these have become established techniques for the production of transgenic plants, since there has been little substantial proof so far of integrative transformation, sustained expression, or inheritance of the transferred genes (Potrykus, 1991).

1.5 *Agrobacterium* as a vector for gene transfer

Agrobacterium is a soil bacterium, the most important species of which cause crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). It has long been known as a plant pathogen, but only relatively recently has the utility of this bacterium as a gene transfer system been recognised. This was first conclusively shown by Chilton *et al.* (1977) who demonstrated that crown galls were produced as a result of the transfer and integration of bacterial genes into the plant genome. Since then it has been established that part of the bacterial Ti (tumour-inducing) or Ri (root-inducing) plasmid's transferred DNA (T-DNA) is transferred into the nuclear genome of plant cells (Fraley *et al.*, 1986). Some of the inserted T-DNA genes encode enzymes responsible for the biosynthesis of phytohormones and/or proteins affecting the sensitivity of plant cells to phytohormones. It is the expression of these genes that results in overgrowths (tumours) and hairy roots. Other T-DNA genes code for enzymes involved in the production and secretion of opines. Opines are amino acid and sugar derivatives which are not normally produced by untransformed plant cells (Tempé and Goldmann, 1982). These are secreted from transformed plant cells into the intercellular regions of a tumour or rhizosphere of a hairy root and the

bacterium uses these as a carbon and nitrogen source (Petit and Tempé, 1985). *Agrobacterium* itself does not appear to express the T-DNA genes (Grant *et al.*, 1991).

The gene-transferring ability of *Agrobacterium* has been exploited to transfer foreign genes into a large number of dicotyledonous plants, including some important crops (for a more detailed review see Gelvin (1990) and Grant *et al.* (1991)), and to a limited number of monocotyledonous plants (Table 1.1). Within the last decade, many workers have used molecular biology technology to manipulate the T-DNA of *Agrobacterium* for the development of gene vectors to produce transgenic plants.

1.5.1 Ti and Ri plasmids

Ti and Ri plasmids are named according to the *Agrobacterium* strain from which they were originally isolated. These plasmids are large in size, ranging from 140 to 235 kilobase pairs (kbp) (Grant *et al.*, 1991). The regions of importance on Ti and Ri plasmids include:

1) the **T-DNA**, bordered by two 25 bp direct repeats at each end - any DNA between these borders is capable of being transferred to the plant cell,
2) the **virulence (*vir*) region**, which encodes genes responsible for excision, transfer and integration of T-DNA into the plant genome, and
3) **opine catabolism genes**, which enable *Agrobacterium* to utilize opines secreted from tumours and hairy roots.

The virulence region of Ti and Ri plasmids occurs outside the T-DNA. It is about 30 kbp long and is organized into seven distinct complementation groups (operons) including *vir* A, *vir* B, *vir* C, *vir* D, *vir* E, *vir* G and *vir* H (formerly *pin* F) (Stachel and Nester, 1986). These operons encode *trans*-acting factors essential for T-DNA transfer. Mutations in the *vir* region generally lower the virulence, i.e. DNA transforming potential, of the inciting bacterium (Zambryski, 1992). Within each of

Table 1.1 Monocotyledonous plants susceptible to *Agrobacterium* and/or from which transgenic plants or transformed cell cultures have been produced

Plant	Response	Reference
Allium cepa (onion)	tumours (opine-positive), hairy root	Dommisse *et al.*, 1990
Anthurium andraeanum	*in vivo* and *in vitro* tumours (opine positive)	Kuehnle and Sugii, 1991
Arthropodium cirratum (New Zealand rock lily)	tumours (opine-positive)	Conner and Dommisse, 1992
Asparagus officinalis	tumours (opine-positive); transformed cell cultures; transgenic plants	Hernalsteens *et al.*, 1984; Bytebier *et al.*, 1987; Conner *et al.*, 1988; Prinsen *et al.*, 1990
Chlorophytum capense	opine-positive swellings	Hooykaas-van Slogteren *et al.*, 1984
Chlorophytum comosum	tumours (opine-positive)	Feng *et al.*, 1988
Cordyline terminalis and *C. rubra*	tumours (opine-positive)	Suseelan *et al.*, 1987
Cordyline australis (NZ cabbage tree)	tumours (opine-positive)	Conner and Dommisse, 1992
Dioscorea bulbifera (yam)	*in vitro* cell cultures; tumours (opine-positive)	Schäfer *et al.*, 1987; Conner and Dommisse, 1992
Gladiolus sp.	opine synthase activity	Graves and Goldman, 1987
Hippeastrum rutilum	tumours (opine-positive)	Feng *et al.*, 1988
Hordeum vulgare (barley)	tumours (opine positive)	Deng *et al.*, 1990
Monstera deliciosa (fruit salad plant)	tumours (opine-positive)	Conner and Dommisse, 1992
Narcissus cv Paperwhite	opine-positive swellings	Hooykaas-van Slogteren *et al.*, 1984
Nerine bowdenii	tumours (opine-positive)	Conner and Dommisse, 1992
Oryza sativa (rice)	transformed cell cultures	Raineri *et al.*, 1990
Polygonatum ×*hybridum* (Solomon's seal)	tumours (opine-positive)	Conner and Dommisse, 1992
Triticum aestivum (wheat)	tumours (opine positive); transformed callus	Deng *et al.*, 1990; Mooney *et al.*, 1991
Tulipa sp. (tulip)	transient expression of GUS gene insert	Wilmink *et al.*, 1992
Zantedeschia aethiopica (arum lily)	tumours (opine-positive)	Conner and Dommisse, 1992
Zea mays (maize)	transient opine synthase activity; transformed plants	Graves and Goldman, 1986; Gould *et al.*, 1991

the operons are varying numbers of open reading frames which are strongly and co-ordinately induced by phenolic compounds leached from wound sites on plants (Grant *et al.*, 1991). One such phenolic compound often used to enhance the frequency of transformation events following inoculation with *Agrobacterium* is the wound response molecule, acetosyringone. This compound was identified as a 'signal' molecule which activated T-DNA transfer to plant cells, by Stachel *et al.* (1985). The structurally similar molecule, α-hydroxyacetosyringone was also identified by Stachel *et al.* (1985). Subsequent work by other groups has since shown that several other phenolic compounds can act as *vir* inducers. These include lignin precursors such as coniferyl alcohol and sinapinic acid (Spencer and Towers, 1988; Melchers *et al.*, 1989; Song *et al.*, 1991), and the methyl ester of syringic acid (Spencer *et al.*, 1990). It has also been shown that non-phenolic compounds, e.g., selected sugars (Shimoda *et al.*, 1990; Cangelosi *et al.*, 1990) and glycine betaine at a low pH (Vernade *et al.*, 1988) can further enhance acetosyringone induction of the *vir* gene. Opines have also been shown to induce the *vir* genes (Veluthambi *et al.*, 1989).

Following induction of *vir* gene expression, molecular reactions occur on the T-DNA element of the Ti plasmid to generate a transferable T-DNA copy (Zambryski, 1992). Firstly, single-stranded (ss) endonucleolytic cleavages are detected between the third and fourth bases of the bottom strand of the 25 bp border repeats (Wang *et al.*, 1987; Albright *et al.*, 1987). These nicks are then used as initiation and termination sites for displacement of a linear ss copy of the bottom strand of the T-DNA region, designated the T-strand (Stachel *et al.*, 1986). These reactions reflect the polarity and functionality of the T-DNA borders, i.e., the T-strand is generated in a right to left direction. After formation of the T-strand, this DNA must traverse the bacterial cell membrane and cell wall, the plant cell wall, and the plant cell and nuclear membranes. Once inside the nucleus, the T-strand must then stably integrate itself into the plant cell genome. Throughout the transfer process, the T-strand must avoid degradation by nucleases. It is thought that the T-strand exists as a DNA-protein complex (the T-complex), in which the DNA is protected from the action of nucleases (Howard and Citovsky, 1990). Evidence also suggests that T-DNA

preferentially integrates at random into DNA sequences that can be transcribed, i.e. single copy DNA (Koncz *et al.*, 1989; Kertbundit *et al.*, 1991; Topping *et al.*, 1991).

1.5.2 The introduction of *Agrobacterium* T-DNA into plant cells

Intentional wounding of the plant to enable *Agrobacterium* infection has become the basis of the most commonly used method to produce transgenic plants via *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation of plant tissue was first achieved by co-cultivation of protoplasts, and subsequent regeneration from these (Horsch *et al.*, 1984; De Block *et al.*, 1984). *Agrobacterium*-mediated transformation using the technique of co-cultivation of protoplasts did, however, have major limitations, some of which included: not all species of plants could be readily regenerated from protoplasts, the entire process could take up to six months from protoplast to plant, and plants derived from protoplasts were more prone to accumulation of mutations or chromosomal abnormalities. Co-cultivation of leaf discs with *Agrobacterium*, a technique which was pioneered by Horsch *et al.*, (1985), was shown to be a much quicker and more efficient method of transferring T-DNA genes into plants. In the original experiments, leaf discs were co-cultivated with co-integrate vector strains of *Agrobacterium* which contained the chimeric *nos*-nptII-*nos* gene, and transformed plants were regenerated directly from leaf discs following selection on kanamycin. Since these results were reported, transgenic plants have been produced from a wide range of species using the leaf disc co-cultivation technique or modifications of it (Grant *et al.*, 1991).

Although *Agrobacterium*-mediated transformation has been highly successful for a number of crop plants, considerable research is still required to establish efficient production regimes for transgenic plants in many other crops. For some crops the problem is the scale-up of transgenic production, especially where only one or at most a few transgenic plants have been produced, e.g. asparagus (Conner *et al.*, 1988), peas (Puonti-Kaerlas *et al.*, 1990), and walnut (McGranahan *et al.*, 1988).

For others, the problem goes beyond this to the point of being unable to produce any transformed plants at all. The latter generally occurs in species which do not form tumours after inoculation with *Agrobacterium*, e.g. cereals. The absence of tumour formation following inoculations with virulent *Agrobacterium* strains has been attributed to a lack of wound response (Potrykus, 1990). It is thought that only those plants and tissues with a pronounced wound response will develop larger populations of wound adjacent cells competent for efficient *Agrobacterium*-mediated transformation.

1.6 Susceptibility of monocotyledonous plants to *Agrobacterium*

Many monocotyledonous plants have for some time been thought to be insensitive to *Agrobacterium* infection and subsequent transformation (De Cleene and De Ley, 1976; De Cleene, 1985). However, considerable work has recently been done to establish a range of monocotyledonous plants as hosts for *Agrobacterium*. Consequently, several monocotyledonous genera have now been reported to respond to *Agrobacterium* inoculation by producing tumours. Published results of tumour responses are summarized in Table 1.1. In these studies, opines or enzymes involved in the synthesis of opines, were detected in extracts of the tumours (or 'swellings', as reported in some cases) which were produced in response to inoculations with wild type strains of *Agrobacterium*. This provided biochemical evidence for *Agrobacterium*-mediated transformation at the cellular level in monocotyledonous plants. On at least one of these species i.e., onions, a pronounced wound response has been observed following both inoculation of bulbs with *Agrobacterium* and injection of bulbs with no bacteria (Dommisse *et al.*, 1990).

As well as resulting in the production of tumours, *Agrobacterium*-mediated gene transfer has also resulted in transient expression of foreign genes in cells of *Tulipa* (Wilmink *et al.*, 1992) and *Zea mays* (Graves and Goldman, 1986), and in stable

integration of the introduced DNA derived from *Agrobacterium*. To date, the only monocotyledonous species from which stably-transformed, transgenic plants have been produced after transformation with *Agrobacterium*, is *Asparagus officinalis* (Bytebier *et al.*, 1987; Conner *et al.*, 1988). Transformed *Triticum* (wheat) cell cultures (Mooney *et al.*,1991) and transformed *Oryza* (rice) (Raineri *et al.*, 1990) cell cultures stably expressing foreign genes have also been produced. All of this work is summarized in Table 1.1.

1.7 Onion (*Allium cepa*) as a host for *Agrobacterium*

Past attempts to infect and transform onion and other *Allium* species with *Agrobacterium* have either been unsuccessful, or at most have been reported as resulting in 'abnormal localized overgrowths' on *A. cepa* bulbs inoculated with *Agrobacterium tumefaciens* (Jakowska, 1949), suggesting that onions may be a host. Because work done in this thesis clearly establishes *A. cepa* as a host for *Agrobacterium*, as evidenced by tumorigenic responses and the production of opines by these tumours, and because a pronounced wound response was evident after inoculation of bulbs with or without *Agrobacterium*, it was thought that the species *A. cepa* could potentially be transformed by *Agrobacterium*. If transformed plants were recovered, onions would join those few monocotyledonous plant species which have already been transformed (Table 1.1). Work done in this project determines the feasibility of developing a transformation system in *A. cepa* utilizing the tumour response of onions to investigate *Agrobacterium*-mediated gene transfer.

1.8 Aims of this project

The first aim of this project was to establish whether onion was a host to

Agrobacterium tumefaciens and/or *A. rhizogenes* (Chapter 2). This was done by inoculating bulbs of selected onion cultivars in different places with several virulent and avirulent *Agrobacterium* strains. The growth of tumours in response to inoculation with virulent *Agrobacterium* strains, and synthesis of opines in these tumours was considered as evidence that bulbs of these cultivars were susceptible to *Agrobacterium* infection, and that T-DNA genes were being expressed.

Tissue culture experiments were conducted concurrently with bulb inoculations to determine the most efficient tissue culture systems for these and other cultivars (Chapter 3). This included induction of callus, regeneration of plants from callus, shoot proliferation and protoplast culture. A tissue culture system was required so that following co-cultivation of explants with *Agrobacterium*, putatively transformed plants could be regenerated from tissue which had survived selection.

Once tissue culture systems were established, dose response experiments with selection agents were performed to determine which concentration of each should be used for selection of transformed cells after co-cultivation (Chapter 4). These experiments were carried out on tissues which would be used as explants in *Agrobacterium* co-cultivation experiments. Kanamycin, geneticin (G418), chlorsulfuron and later, hygromycin were the four selective agents chosen.

With the aim of producing a transformed onion plant or plants, transformation experiments (Chapter 5) were carried out on onions using a number of wild-type and disarmed *Agrobacterium* binary vector strains. Two different binary vectors, *viz* pKIWI110 and pGA643, were used in these experiments. Large numbers of different explant types were co-cultivated with log phase cultures of *Agrobacterium*. In addition to this, basal plates of freshly sterilized onion bulb explants and subcultured *in vitro* shoot cultures were injected with log phase cultures of *Agrobacterium*. Plantlets or callus showing resistance to the appropriate selection agent were assayed for the expression of the proteins NPTII and GUS where appropriate. Southern analyses were also carried out on this tissue.

Chapter 2: Onion is a monocotyledonous host for *Agrobacterium* [1]

2.1. Abstract

Onion (*Allium cepa*) bulbs and leaves were inoculated with 25 virulent strains of *Agrobacterium*. Eleven strains of *Agrobacterium tumefaciens*, one of *A. rubi* and six of *A. rhizogenes* induced tumorous growths at the base of bulbs of the New Zealand-bred cultivars 'Pukekohe Longkeeper' and 'Early Longkeeper'. One *A. rhizogenes* strain, HRI produced a root-like structure arising from a tumour at the base of an 'Early Longkeeper' bulb. The majority of these tumours produced nopaline, suggesting that transformation had occurred at the cellular level. By contrast, nopaline or octopine were never detected in extracts of tumour-free inoculation sites which had been inoculated with virulent or avirulent strains. Tumours appeared earlier on bulbs inoculated with *Agrobacterium* cultured in the presence of acetosyringone. The tumour response to *Agrobacterium* inoculations was genotype-dependent, as only two of four inoculated cultivars produced tumours. After being excised and surface-sterilized, some of these tumours produced roots in sterile culture. The *Agrobacterium* strains which induced the root-producing tumours comprised wild-type strains as well as some harbouring the binary vector pKIWI110. Although tumour-roots were transferred to a range of media, no further callusing or shoot production was seen in the excised tumours.

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[1] An earlier version of the tumour work presented in this chapter has been published in Plant Science 69 (Dommisse *et al.*, 1990). A reprint of this paper is enclosed at the back of the thesis.

2.2. Introduction

Monocotyledonous plants have not traditionally been considered as hosts for *Agrobacterium* infection, with only small tumours or swellings being occasionally recorded in a few species following inoculation with *Agrobacterium* (De Cleene and De Ley, 1976; De Cleene, 1985). However, recent increased interest in using *Agrobacterium* to genetically engineer plants, has resulted in more thorough investigations into the host range of *Agrobacterium* in monocotyledonous plants. A number of monocotyledonous genera have now been reported as hosts for tumour induction by *Agrobacterium*. These are noted in detail in section 1.6 and in Table 1.1.

Crown gall tumours result from the overproduction of the plant growth regulators (PGRs) auxin and cytokinin, which are encoded by T-DNA genes of virulent *A. tumefaciens* strains. Depending on the strain of *Agrobacterium* used, the morphology of the tumours is typified by either the production of amorphous, unorganized callus or by teratomas containing aberrantly organized stem and leaf-like structures (Gelvin, 1990). Other factors, including the host plant species and even the position of inoculation on the plant can determine the incidence of tumour production (Conner and Dommisse, 1992)[2].

The presence of opines in tumours of plants which have been inoculated with virulent strains of *Agrobacterium*, is generally taken to indicate that the tissue has been genetically transformed (Firmin, 1990) (section 1.5). Interest in opines developed after the discovery that their synthesis in crown gall tumours (and, as later found, in hairy roots) is strain-specific (Goldmann *et al.*, 1968; Petit *et al.*, 1970) and that they can be degraded with the same specificity by *Agrobacterium* (Petit *et al.*, 1970). This implied that there was a nutritional relationship between *Agrobacterium* and the diseased tissue of its host. Subsequently, a theory, known as the **opine concept** was

[2] A proof of this paper is enclosed at the back of this thesis

proposed, describing the role of opines as nutritional mediators of parasitism (Petit and Tempé, 1985). The opine concept states that

(i) opines are trophic mediators of parasitism, and

(ii) this function is essential for survival and propagation of Ti and Ri plasmids.

According to this theory, every one of these plasmids should carry genes involved in opine synthesis in transformed plant cells and opine degradation by bacterial cells harbouring the plasmid (Davioud *et al.*, 1988)

Opines produced by *A. tumefaciens* tumours or *A. rhizogenes* hairy roots are generally classified into groups, based on their opine type (Grant *et al.*, 1991). Currently, Ti plasmids are represented by five opine types. These include the octopine, nopaline, agropine, succinamopine and the 'grapevine' types. Ri plasmids are represented by three opine types, including the agropine, mannopine and cucumopine types. A detailed summary table of this information can be found in a review by Grant *et al.* (1991). Two commonly observed opines are nopaline (a condensation product of L-arginine and α-ketoglutarate) and octopine (a condensation product of L-arginine and pyruvate). The chemical structures of nopaline and octopine can be found in the publication of Petit and Tempé (1978). These opines can be detected after TLC electrophoresis using Sakaguchi's reagent, which is specific for compounds containing a guanidine group (Sakaguchi, 1950). Such compounds are usually limited to octopine, nopaline and arginine in plants (Shaw *et al.*, 1988). Both octopine and nopaline stain pink, whilst arginine stains an orange-pink colour. Sakaguchi's reagent has since been used by some groups to test for the presence of nopaline and octopine (Firmin and Fenwick, 1978; Petit and Tempé, 1978; Dahl and Tempé, 1983; Shaw *et al.*, 1988). After staining with this reagent, opine levels can be quantified by scanning densitometry, which enables detection of octopine and nopaline down to levels as low as 1 μg (Shaw *et al.*, 1988).

In vitro studies of axenic tumours have shown that this tissue can grow on hormone-free media (Rudelsheim *et al.*, 1987). Hernalsteens *et al.* (1984) isolated tumour tissue from the monocotyledon *Asparagus officinalis* after infecting stem fragments with the wild-type *A.tumefaciens* strain, C58. This tumour tissue grew *in vitro* on

a hormone-free medium and the T-DNA-encoded opines, nopaline and agrocinopine were detected in these cultures. The tumour lines were subsequently shown by Southern analysis to contain T-DNA segments identical to the T-DNA found in dicotyledonous plants (Bytebier *et al.*, 1987). Axenic tumour tissues capable of proliferation on hormone-free media, and of producing opines, have since been isolated from other monocotyledonous plants, including *Anthurium andraeanum* (Kuehnle and Sugii, 1991), *Dioscorea bulbifera* (Schäfer *et al.*, 1987), and *Oryza sativa* (Raineri *et al.*, 1990). Transfer of *Agrobacterium* T-DNA to the cells of these species has been confirmed by Southern hybridization (Schäfer *et al.*, 1987; Raineri *et al.*, 1990; Kuehnle and Sugii, 1991). The work described in the following chapter establishes onion as a host for *Agrobacterium* and provides substantial evidence for the expression of T-DNA genes. Responses of excised tumours in axenic culture are also described.

2.3. Materials and Methods

2.3.1 Induction of tumours

Onion (*Allium cepa* cvs Pukekohe Longkeeper (PLK), Early Longkeeper (ELK), Southport White Globe (SWG) and Japanese Saporo Yellow (JSY)) bulbs were inoculated with 27 strains of *Agrobacterium* comprising 16 strains of *A. tumefaciens*, 10 strains of *A. rhizogenes* and one strain of *A. rubi* (Table 2.1). Strains LBA4404 and K1, which are incapable of inducing tumours, were used as bacterial controls. All strains of *Agrobacterium* were grown on solid AB medium (Chilton *et al.*, 1974) plus 0.5% (w/v) yeast extract and 20 mgl^{-1} cycloheximide at 28°C.

Bulbs were inoculated by stabbing with sterile needles dipped in *Agrobacterium* colonies. Inoculation sites included the base of the bulb (close to the basal meristem), around the equator of the bulb, near the neck of the bulb and in sprouted

Table 2.1 Tumour responses and opine production of tumours, following inoculation of 'Pukekohe Longkeeper' (PLK) and 'Early Longkeeper' (ELK) bulbs with 27 strains of *Agrobacterium*.

Strain	[b]Ti/Ri plasmid	Opine type	Tumour response (cultivar)	[d]Opine detected (mg/g FW)
A. tumefaciens				
LBA4404	pAL4404	null	-	0.000
[a]K1	null	null	-	0.000
B6	pTiB6	octopine	-	0.000
H100	pTiH100	nopaline	-	0.000
[a]A281	pTiBo542	agropine	+ PLK, ELK	-[c]
[a]A722	pTiA6NC	octopine	+ PLK, ELK	trace (ELK)
[a]C58	pTiC58	nopaline	+ PLK, ELK	0.184 (ELK)
6025	UCTi	nopaline	+ PLK	0.473
6675	UCTi	nopaline	+ PLK	0.250
8302	UCTi	nopaline	+ PLK, ELK	0.826 (PLK)
8317	UCTi	nopaline	+ PLK, ELK	trace (PLK)
8326	UCTi	nopaline	+ PLK	0.462
8330	UCTi	nopaline	+ PLK, ELK	0.410 (ELK)
8375	UCTi	nopaline	-	0.000
8367	UCTi	nopaline	+ ELK	0.881
[a]A4T	pRIA4	agropine	+ PLK, ELK	-[c]
A. rhizogenes				
A4	pRIA4	agropine	-	-[c]
HRI	pRiHRI	agropine	+ PLK	-[c]
TR7	pRiTR7	mannopine	-	-[c]
TR101	pRiTR101	mannopine	-	-[c]
TR105	UCRi	unknown	+ PLK	-[c]
TR107	UCRi	mannopine	-	-[c]
1855	pRi1855	agropine	+ PLK	-[c]
8196	pRi8196	mannopine	+ ELK	-[c]
15834	pRi15834	agropine	+ PLK	-[c]
11325	UCTi	nopaline	+ PLK, + ELK	0.957 0.566
A. rubi				
13335	UCTi	octopine	+ ELK	trace

[a] These strains have the same C58 chromosomal background but differ in plasmid content.

[b] UCTi = uncharacterized Ti plasmid; UCRi = uncharacterized Ri plasmid.

[c] = Opine analysis not performed (see text).

leaves. Inoculated and control bulbs were placed on top of empty beakers and left at room temperature (20-24°C) for up to 50 days. Six or seven weeks after inoculation, tumorous growths at basal inoculation sites, and swellings at bulb equator inoculation sites were excised. The same regions were cut from the inoculated controls (K1 and LBA4404) and the uninoculated controls.

2.3.2 Opine analysis

Analysis of opines was carried out on tumour and control tissue by electrophoresis on cellulose thin layer plates and staining with Sakaguchi's reagent, as outlined in the protocol of Shaw *et al.* (1988). Known amounts of nopaline and octopine standards were spotted onto the cellulose with and without control inoculation extract ('PLK' inoculated with LBA4404). This step was carried out to check if sugars in the bulb extract were causing retention of the migration of standards. Slowing down of migration was sometimes apparent, as spots which stained the pink colour of the nopaline standard did not migrate as far as the nopaline standard.

To enable direct comparison of opine-positive spots on the electrophoretogram, each volume of sample spotted was extracted from the same weight of tumour, wound response or control bulb tissue. To determine the opine types of strains with uncharacterized Ti or Ri plasmids (section 2.4.2; Table 2.1), these strains of *Agrobacterium* were plated on AB medium without NH_4Cl or glucose. Nopaline, octopine or mannopine were included in the medium as the sole source of carbon and nitrogen. Nopaline and octopine staining patterns of extracts from tumours induced by these strains were used to confirm results.

2.3.3 Effects of acetosyringone on tumour induction

To test the effect of acetosyringone on tumour formation and T-DNA expression, *Agrobacterium* strains were cultured overnight (25°C, dark, gyratory shaker, 150 rpm) in MG/L broth (Garfinkel and Nester, 1980). Each strain was cultured with and without 20 μM acetosyringone (Sheikholeslam and Weeks, 1987). A total of

1 ml of bacterial suspension was inoculated with a sterile syringe into several sites at the base of the bulb.

2.3.4 *In vitro* culture of tumour tissue

'Pukekohe Longkeeper' onion bulbs were inoculated with several wild-type *Agrobacterium* strains (section 2.3.1), and with virulent and avirulent strains which harboured the binary vector pKIWI110 (Janssen and Gardner, 1989; see Fig 5.1 for detail of the T-DNA region). Four to six weeks after inoculation of bulbs, tumours had reached about 4-5 mm in diameter. At this stage the tumours were excised and surface-sterilized for *in vitro* studies. The parts of the bulb which were inoculated with the avirulent *Agrobacterium* strains LBA4404 and K1, and those stabbed only with a sterile needle, were also excised and surface-sterilized in the same way. Bulbs were stripped of their outermost scale/s, wiped with 95% ethanol, and tumours excised with a scalpel dipped in 95% ethanol. Excised tumours were surface-sterilized by immersion in a 25% v/v solution of commercial bleach (5% w/w sodium hypochlorite), with stirring, for 2-3 minutes. They were then rinsed 3-4 times in sterile distilled water. All tumours, including those produced after inoculation with virulent strains containing pKIWI110, were excised and cultured as described below.

Tumours were initially placed in the dark (25°C) on two types of media:

1. BDS (Dunstan and Short, 1977), the basal medium used for callus production, seed germination, regeneration and seedling clonal propagation (section 3.3), and
2. onion shoot proliferation basal medium (with 0.12 mgl^{-1} NAA and 2 mgl^{-1} BA added, see section 3.3.4).

Half of the root-producing tumours which had resulted from inoculations with the virulent strains C58 and A4T harbouring the binary vector pKIWI110, were excised and cultured on the above media supplemented with 750 mgl^{-1} kanamycin. In addition, half of the non-root-producing tumours induced by strain A722 containing pKIWI110, were also cultured on identical kanamycin-supplemented media.

Neither media used contained PGRs. Since browning of tumours sometimes occurred, *in vitro*-cultured tumours were subsequently transferred to the same media with lowered macro-, micro- and iron salts concentrations. These media contained 1/8× macro-, micro- and iron salts, and half the concentration of vitamins normally present in full-strength media. Previous work on non-transformed onion roots growing in culture has shown that the roots grow better in media containing as little as 1/10× concentration of basal salts (Dr J D Ferguson, pers. comm.). Both low strength media were made up with PGRs (0.045mgl^{-1} NAA, 1 mgl^{-1} BA) and without PGRs. These were added in an attempt to stimulate the growth of those tumours which had not responded on the hormone-free media. Some root-producing tumour cultures were transferred to the light (25°C, 16 h day, 30 μE/m^2/sec).

To check for the presence of contaminating, surface-living bacteria, roots which grew from tumours were smeared across nutrient agar plates or were dipped into liquid nutrient broth, both of which contained 0.5% (w/v) yeast extract. Nutrient media were incubated overnight at 28°C (agar plates), and 25°C (nutrient broth) with shaking.

2.3.5 Opine production by tumour roots

Roots produced by tumours resulting from inoculations with wild-type *Agrobacterium* strains HRI and 8367, and with strains C58, A4T and 6675 harbouring the binary vector pKIWI110, were extracted and analysed for opines using the method of Shaw *et al.* (1988).

2.3.6 β-glucuronidase assays on tumour roots

β-glucuronidase (GUS) histochemical assays (Jefferson, 1987) were carried out on roots growing from tumours incited by *A. tumefaciens* strain A4T, harbouring the binary vector pKIWI110. Roots from aseptic onion shoot cultures were used as controls. To check that GUS activity was not bacterial in origin, roots were smeared across nutrient agar plates and dipped in liquid nutrient broth, both media containing

yeast extract (0.5%). Nutrient media were incubated overnight at 28°C (agar plates), and 25°C (nutrient broth) with shaking.

2.4. Results

2.4.1 Onion tumours

Three weeks after inoculation, small tumours were visible at inoculation sites close to the basal meristem of some of the 'PLK' and 'ELK' bulbs which had been inoculated with virulent strains of *Agrobacterium tumefaciens* and *A. rhizogenes* (Table 2.1). They appeared as white cell masses immediately surrounding the inoculation sites (Plate 2.1). These tumours continued to steadily increase in size until they were excised for opine analyses after 6-7 weeks. A higher percentage of tumours resulted from inoculations of mature 'PLK' and 'ELK' bulbs which were carried out two months after harvesting, than from immature bulbs which had been harvested two months earlier than usual. Not all virulent strains induced tumorous growths, and these growths were consistently absent from the inoculated (LBA4404, K1) and uninoculated controls (Table 2.1). No tumours were seen at the basal inoculation sites of 'SWG' or 'JSY' bulbs. Swollen, watery tissue, thought to be a wound response, was visible around basal inoculation sites of bulbs of all four cultivars inoculated with avirulent *Agrobacterium* strains, and around the wounds of uninoculated controls. This tissue was also present at sites inoculated with virulent strains which did not cause the described tumour results (Table 2.1).

When inoculated into the base of 'ELK' bulbs, one strain of *A. rhizogenes*, HRI, induced a hairy root-like response which developed from what appeared to be a tumour (Plate 2.2). All other *A. rhizogenes* strains used induced tumours like those seen with *A. tumefaciens*. In bulbs of all four cultivars, including controls, watery swellings occurred at the equatorial inoculation sites (Plate 2.3). No tumours or noticeable swellings were seen at the neck of the bulb or on inoculated leaves of onions.

2.4.2 Opines in tumour tissue

Eleven strains of *A. tumefaciens*, six strains of *A. rhizogenes* and one strain of *A. rubi* used, induced some tumour response in inoculated 'ELK' and 'PLK' bulbs (Table 2.1). Only tumours from octopine- or nopaline-producing strains were analysed for these opines. Other types of opines were not determined as the method commonly used for analysing agropine and mannopine (Petit *et al.*, 1983) was not considered specific enough to detect only these two compounds in tumours without also detecting other reducing sugars which co-migrated during electrophoresis.

Eleven strains of *Agrobacterium* induced opine-positive tumours on either or both of the onion cultivars (Table 2.1). Some of the tumour extracts which were subjected to electrophoresis and staining are shown in Plate 2.4. Variation between amounts of nopaline produced by different tumours was apparent from densitometry readings. Strains 8302, 8367 and 11325 consistently produced the highest amounts of nopaline (Table 2.1). Sample migration was occasionally retarded by sugars in the extract. This retention can be seen in the tumour extract of 'PLK' inoculated with *A. tumefaciens* strain 6025 (Plate 2.4, lane B). No opines were detected in extracts of 'JSY' and 'SWG' bulb base inoculation sites after inoculations with virulent or avirulent strains of *Agrobacterium*.

One octopine- and one nopaline-producing strain of *A. tumefaciens* produced tumours on 'PLK' and 'ELK' bulbs which only contained trace amounts of either opine type, even when higher levels of extract (10-20 μl) were spotted onto electrophoresis plates (Table 2.1). However, detection of opines in samples applied at these levels was more difficult, as high sugar levels made the samples viscous, resulting in suboptimal migration. Trace amounts of nopaline were also detected in the bulb equator wound response swellings of 'PLK' bulbs inoculated with *A.tumefaciens* strain 6025. However, nopaline was not detected in the bulb equator wound swellings resulting from any other inoculations. Quantitation of nopaline from the 6025 wound response swellings was not possible, as amounts were below the detection limit of densitometry.

A.tumefaciens strains for which opine types had not yet been determined (6025, 6675, 8302, 8317, 8326, 8330, 8367 and 8375) were shown by growth on restricted media and/or by electrophoretic staining patterns, to be nopaline strains. Growth of bacteria on a medium supplemented only with nopaline indicated that these *Agrobacterium* strains each produce enzymes involved in the catabolism of nopaline.

2.4.3 Effects of acetosyringone on tumour production

Bulbs which were inoculated with *Agrobacterium* cultured in the presence of acetosyringone, consistently formed tumours after two weeks, at least a week earlier than in non-acetosyringone treatments. However, acetosyringone had no effect on the final frequency of tumours, and did not result in tumour production with ineffective *Agrobacterium* strains (data not shown).

2.4.4 Roots produced by *in vitro*-cultured tumours

Tumours which had been excised from inoculated bulbs usually produced roots within 3-4 weeks of being established in culture. No further callus was produced by tumour explants. Tumours producing roots in culture included those from 'PLK' bulbs inoculated with *A. tumefaciens* strains 8367 and C58, and *A. rhizogenes* strain HRI, and with the *A. tumefaciens* strains C58, A4T and 6675, each of which was harbouring the binary vector pKIWI110. Other excised tumours did not produce roots in culture. Results of inoculation/ *in vitro* culture combinations are shown in Table 2.2.

Tumours produced 1-6 roots in culture (Plate 2.5; Table 2.2). Some of these roots greened when cultures were transferred to the light. Roots continued to elongate when transferred to basal media containing NAA and BAP, but no new roots were produced. No bacteria grew on the plates across which the roots had been smeared, or in the liquid broth into which roots had been dipped. Excised control inoculations i.e., those inoculated with an avirulent bacterial strain or with no bacteria, did not respond to *in vitro* culture. Shoots or plantlets could not be regenerated from the *in vitro* tumour roots. After 8-10 months

Table 2.2 Production of roots by excised, *in vitro*-cultured tumours, and responses of some of these roots when grown on kanamycin.

Agrobacterium strain	Total no. roots produced by excised tumours	Growth on kanamycin (750 mgl^{-1})
pKIWI110/A281	0	n.a.[1]
pKIWI110/C58	6	root elongation (no new roots produced)
pKIWI110/A722	0	no growth of excised tumour
pKIWI110/A4T	4	root elongation (no new roots produced)
pKIWI110/6675	5	n.a.
pKIWI110/8330	0	n.a.
pKIWI110/8302	0	n.a.
C58	1	no binary vector (n.a.)
6025	0	" " " (n.a.)
HRI	2	" " " (n.a.)
8367	5	" " " (n.a.)

[1] n.a. = not attempted

of regular subcultures onto the media specified in section 2.3.4, tumour roots stopped growing. Roots which had grown from those excised tumours resulting from inoculations with binary vector strains of *Agrobacterium*, elongated when placed on kanamycin-supplemented media, but no further roots were produced on these media (Table 2.2). No callus growth was seen when excised tumours from binary vector strain inoculations were plated on media with kanamycin (Table 2.2).

2.4.5 GUS histochemical assays on root tissue

Roots produced by tumours resulting from inoculations with the *Agrobacterium* strain A4T harbouring pKIWI110, were shown by the histochemical assay to have GUS activity. This activity was localized to the vascular region of the root (Plate 2.6). Blue precipitate was also visible in roots of some of the control plantlets assayed for GUS activity.

2.4.6 Opine analysis of root tissue

Opine analysis showed large amounts of arginine to be present in roots growing from tumours and in control roots. However, nopaline and octopine were not detected in any of the samples analysed.

2.5. Discussion

Although there have been some reports of monocotyledonous species developing tumour-like swellings at sites of *A. tumefaciens* inoculation (De Cleene and De Ley, 1976; De Cleene, 1985), control inoculations to distinguish between general wound responses and tumour induction have not usually been performed. This is clearly important in *A. cepa* which produced a marked general wound swelling at all of the inoculation sites around the equator of the bulb (Plate 2.3). These wound swellings were visible on bacterial and non-bacterial controls. As these swellings did not look

any different to those produced at control inoculation sites, it was initially assumed that they were only wound and not tumour responses. However, the presence of trace amounts of nopaline detected in these swellings produced by inoculation of 'PLK' with the virulent strain 6025, indicated that the *Agrobacterium* present in the wound swellings incited small opine-producing tumours in the inoculation sites.

Biochemical evidence for *Agrobacterium*-mediated transformation of cells in tumours on monocotyledonous species has been established in most cases. Tumours and small tumour-like external swellings induced by *A. tumefaciens* have been shown to produce opines in a number of species including *Anthurium andraeanum* (Kuehnle and Sugii, 1991), *Arthropodium cirratum* (Conner and Dommisse, 1992), *Asparagus officinalis* (Conner *et al.*, 1988), *Chlorophytum comosum* (Feng *et al.*, 1988), *Cordyline australis* (Conner and Dommisse, 1992), *C. terminalis*, *C. rubra* (Suseelan *et al.*, 1987), *Dioscorea bulbifera* (Conner and Dommisse, 1992), *Hippeastrum rutilum* (Feng *et al.*, 1988), *Hordeum vulgare* (Deng *et al.*, 1990), *Monstera deliciosa*, *Nerine bowdenii*, *Polygonatum* ×*hybridum* (Conner and Dommisse, 1992), *Triticum aestivum* (Deng *et al.*, 1990) and *Zantedeschia aethiopica* (Conner and Dommisse, 1992). Opine synthase activity has also been detected at the inoculation sites of *Agrobacterium* in *Zea mays* (Graves and Goldman, 1986), and *Gladiolus* (Graves and Goldman, 1987), although distinct tumour responses were not observed in *Zea* or *Gladiolus*.

This study provides evidence that *Allium cepa* is a monocotyledonous host for *Agrobacterium tumefaciens* transformation. Results presented here also show that onions are a host for *A. rhizogenes* and *A. rubi*. The genus *Allium* is usually placed in the Liliaceae, or sometimes in the segregate family, Alliaceae. Five of the other 21 monocotyledonous species for which there is good evidence for *Agrobacterium* transformation (Table 1.1.), *viz Asparagus, Chlorophytum capense* and *C. comosum*, *Polygonatum* and *Tulipa*, are also in genera usually placed in the Liliaceae. In total, 10 of the 22 species (including *Allium*) belong in families classified into the order Liliales. Based on these results, one could predict that more plants in the Liliales order might be susceptible to *Agrobacterium*-mediated transformation.

A marked contrast was observed between *Agrobacterium* strains A4T (consistent tumour induction in 'PLK' and 'ELK') and A4 (consistently no response in these two genotypes). Since both of these strains contain the same Ri plasmid (pRiA4) and differ only in their chromosomal backgrounds, this contrast can be attributed to chromosomal effects. All other virulent *Agrobacterium* strains with the same chromosomal background as A4T (C58, A722, A281) also induced tumours in both onion genotypes.

It has been shown that opines may be formed from arginine in wild-type plant tissue growing on media supplemented with arginine (Christou *et al.*, 1986). Therefore, detection of opines may not always be absolute proof for *Agrobacterium*-mediated transformation of cells. For this reason, uninoculated tissue was analysed in the same way as tumourigenic and non-tumourigenic inoculated tissue. Neither octopine nor nopaline was detected in any uninoculated tissue. Quantitation of accumulated opines in onion tumours by scanning densitometry, showed levels to be at least one hundred times more than the minimum detectable level (Table 2.1). In contrast, no densitometric readings were recorded from electrophoretograms of inoculated or uninoculated control tissue.

Acetosyringone reduced the time taken for *Agrobacterium*-induced tumours to develop in onions, although it had no effect on the ultimate frequency of tumour appearance, or on the range of *Agrobacterium* strains that were effective. Acetosyringone and related compounds are known to induce the expression of *Agrobacterium* virulence genes (Stachel *et al.*, 1985; Bolton *et al.*, 1986) and the resulting circularization of T-DNA (Usami *et al.*, 1987). These effects may have increased the frequency of transformed cells at the wound site, causing tumours to appear earlier. Usami *et al.* (1987) demonstrated an absence of these *vir*-inducing plant compounds in *Allium fistulosum* and in other monocotyledonous plants, and showed that supplying acetosyringone overcame this limitation for T-DNA circularization. They suggested this as a reason why *Agrobacterium*-induced tumour formation is often blocked in monocotyledonous plants. However, subsequent work by the same group has since shown that wheat and oats contain a substance(s) that

induces *vir* gene expression (Usami *et al.*, 1988). In this study, it has been established that provided the correct *Agrobacterium* strains are used, tumours consistently form on onions, even in the absence of acetosyringone or other exogenously-supplied *vir*-inducing compounds.

It is likely that the tumourigenic and rootlike responses seen in these inoculations were mainly localized in the area of the bulb immediately surrounding the basal meristem since these cells are younger and likely to be more metabolically active than cells further from the meristem. The swellings seen in inoculations around the equator of the bulb were assumed to be wound responses for two reasons: firstly, they were visible within 48 hours of inoculation and secondly, excepting one case where a trace amount of nopaline was found in one of these swellings ('PLK' inoculated with strain 6025), no nopaline or octopine was detected in these swellings.

An observation earlier this century that 'localized abnormal overgrowths' occurred on onion bulbs inoculated with *Bacterium (sic) tumefaciens* (Jakowska, 1949) suggested that *Agrobacterium* could infect and transform *A. cepa* cells. What was then thought to be susceptibilty of *Allium cepa* to *A. tumefaciens* has been confirmed by the work presented here.

2.5.1 Root production from *in vitro*-cultured tumours

The lack of regenerative capacity of onion tumour tissue has also been commonly observed in other plants. Gelvin (1990) reported that crown gall tumours rarely revert to tissue capable of regenerating plants, although hairy roots of various species can spontaneously regenerate plants. Although roots were spontaneously regenerated from *in vitro*-cultured tumours, opines were not detected in these roots. The tumours from which roots grew were probably opine positive, given that previous inoculations of the same cultivars with the same *Agrobacterium* strains had resulted in opine-positive tumours. This result does not necessarily mean that the roots were not producing opines, but possibly that given the small amounts of tissue available for analysis, opines were not present in levels high enough to be detected by the

technique used. Similar results have been reported for *Polygonatum* ×*hybridum* and *Zantedeschia aethiopica*, where tumours were small and did not enlarge with age (Conner and Dommisse, 1992). However, it is also possible that the tumour explants established in culture were chimeric tissues consisting of transformed and untransformed cells. Consequently, roots may have developed from untransformed cells, in response to PGRs being cross-fed from neighbouring transformed tumour cells.

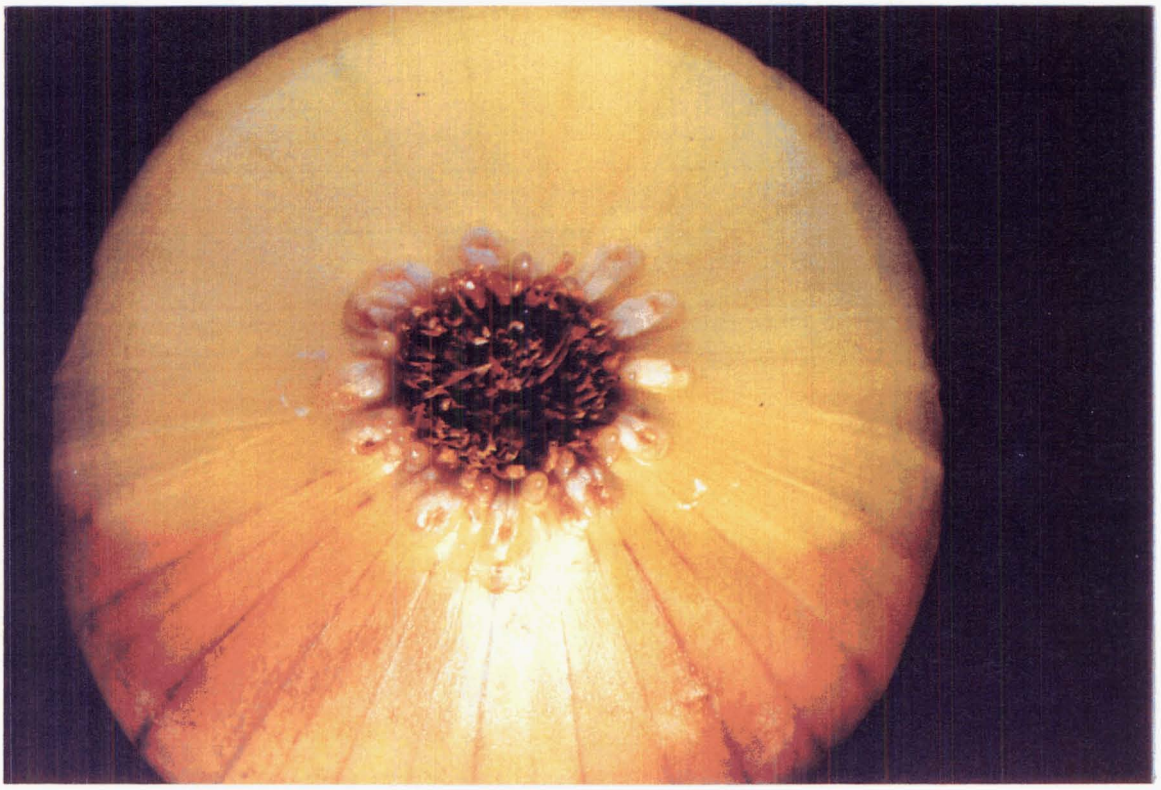
The reason for lack of rapid growth of tumour cells on the hormone-free medium is unclear. Other workers have shown that excised *Asparagus* tumour tissue grew only slowly as hard compact calli on hormone-free media and that growth of these calli was not enhanced unless they were transferred to media containing PGRs (Conner *et al.*, 1988). However, the growth of excised onion tumour tissue was not enhanced after transfer to media containing PGRs. It is possible that onion tumour cells were sensitive to the ethanol and hypochlorite used to sterilize excised tumours, and that their subsequent growth on the medium may have been inhibited for this reason.

The GUS-positive response of tumour- and control roots, i.e. transformed and untransformed tissue, has recently been investigated by other workers. Hu *et al.* (1990) uncovered the reason why many workers had been troubled by 'false positives' or 'background' activities with GUS assays. They surveyed several organs of a range of 52 seed plants, including scallion (*Allium fistulosum*), and found that *A. fistulosum* tissues had intrinsic GUS-like activity as detected by the histochemical and flurometric assays. These assays were, however, not carried out under sterile conditions and it is possible that bacterial enzymes may have also contributed to this GUS-like activity. A modified GUS histochemical assay protocol (Kosugi *et al.*, 1990), which includes the addition of 20% methanol to the reaction buffer, has since enabled intrinsic GUS-like activity to be suppressed (section 5.3.3). Expression of the introduced gus gene is reported not to be suppressed under these conditions. Twenty percent methanol was not included in the GUS assay reaction buffer when onion tumour root tissue was being assayed, as the technique had not been published

at this stage of the project. By the time the (methanol) method was in use, there were no surviving tumour roots to assay.

Following this work, co-cultivations of *Agrobacterium* with onion tissue and *in vitro* injection experiments have been carried out in an attempt to produce transgenic plants (section 5.3). Co-cultivation of basal meristem tissue with *Agrobacterium* is most likely to be the best approach for transformation of onions because this is the tissue type susceptible to *Agrobacterium* when bulbs are inoculated *in vivo*, and onion plants are readily cultured *in vitro* from this explant source (Hussey and Falavigna, 1980).

Plate 2.1 Tumours induced on onion ('Pukekohe Longkeeper') bulb tissue after inoculation with *Agrobacterium tumefaciens* strain C58 (top) and an avirulent control strain, LBA4404 (bottom).



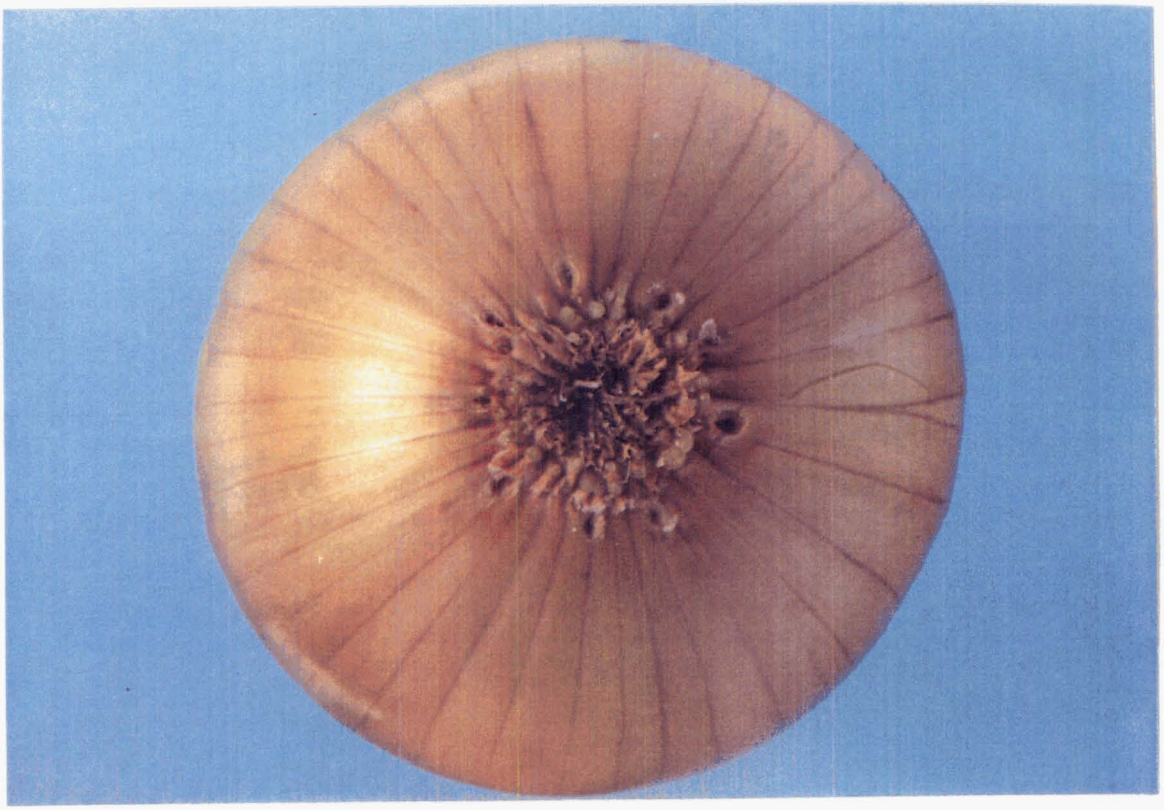
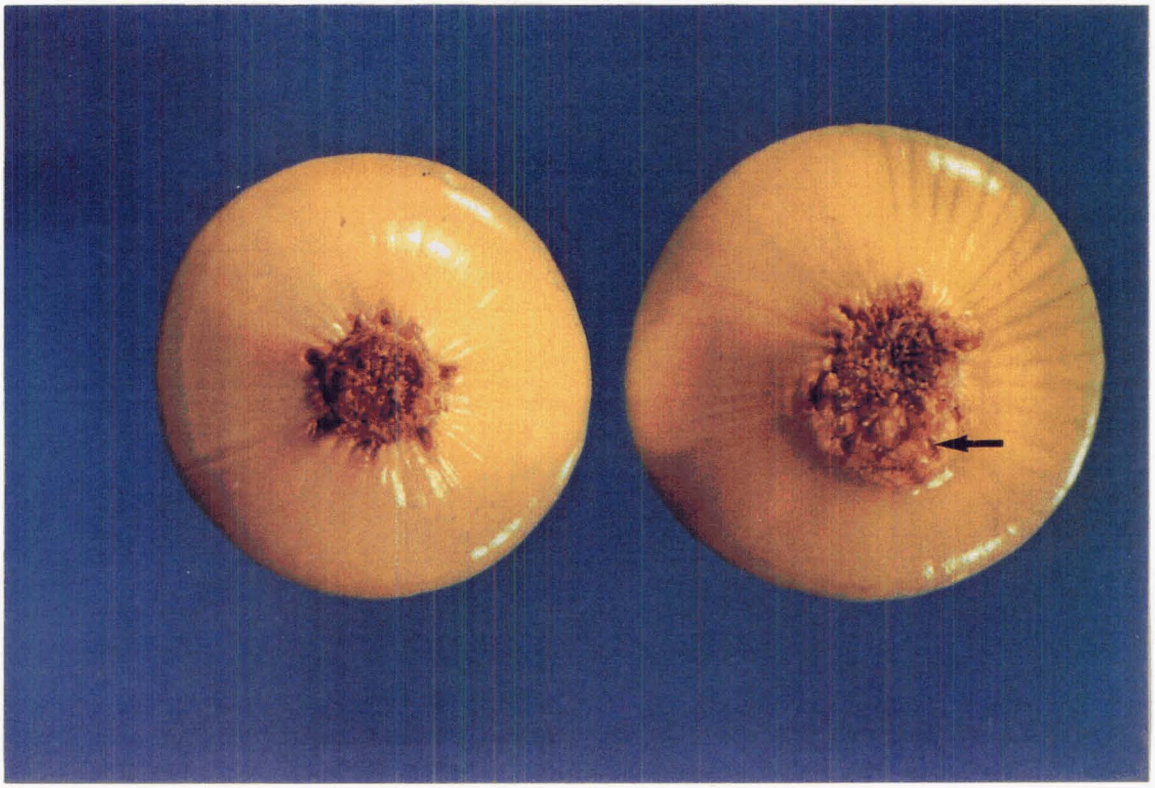


Plate 2.2 Root-like response (arrow) of onion ('Early Longkeeper') bulb inoculated with *Agrobacterium rhizogenes* strain, HRI, (right) and no response after inoculation with an avirulent strain of *A. tumefaciens* LBA4404 (left).

Plate 2.3 Generalized wound response of onion ('Pukekohe Longkeeper') following inoculation with **A**, strain A4T (virulent); **B**, no bacteria; **C**, strain 8317 (virulent); **D**, strain LBA4404 (avirulent).



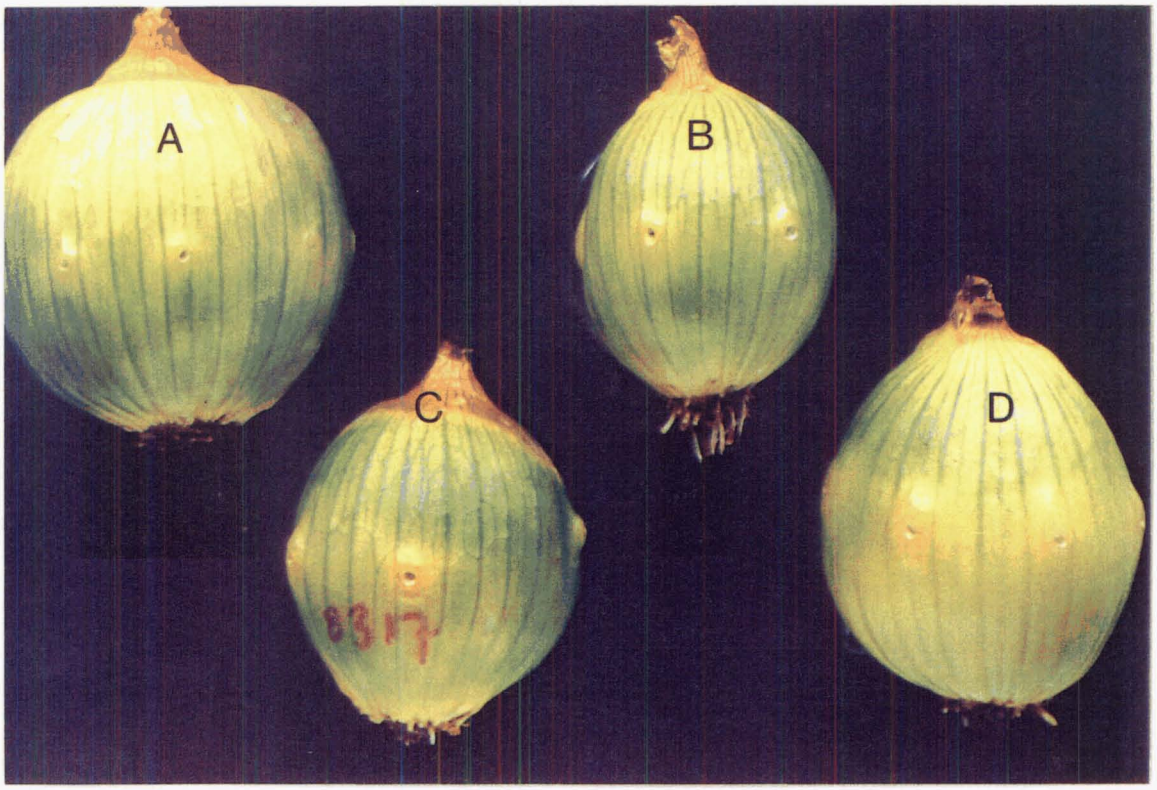

A
B
C
D

Plate 2.4 Opine analyses of onion tumour tissue. Extracts of *Agrobacterium*-induced tumours and control tissue of onion bulbs were subjected to electrophoresis on cellulose thin layer plates and stained with Sakaguchi's reagent. **A**, strain LBA4404 (control), 'PLK'; **B**, strain 6025, 'PLK'; **C**, strain 8302, 'PLK'; **D**, octopine (o) and nopaline (n) standards (2 μg each); **E**, strain 11325, 'PLK'; **F**, strain 11325, 'ELK'; **G**, strain 13335, 'PLK'. Samples run from anode (+) to cathode (-).

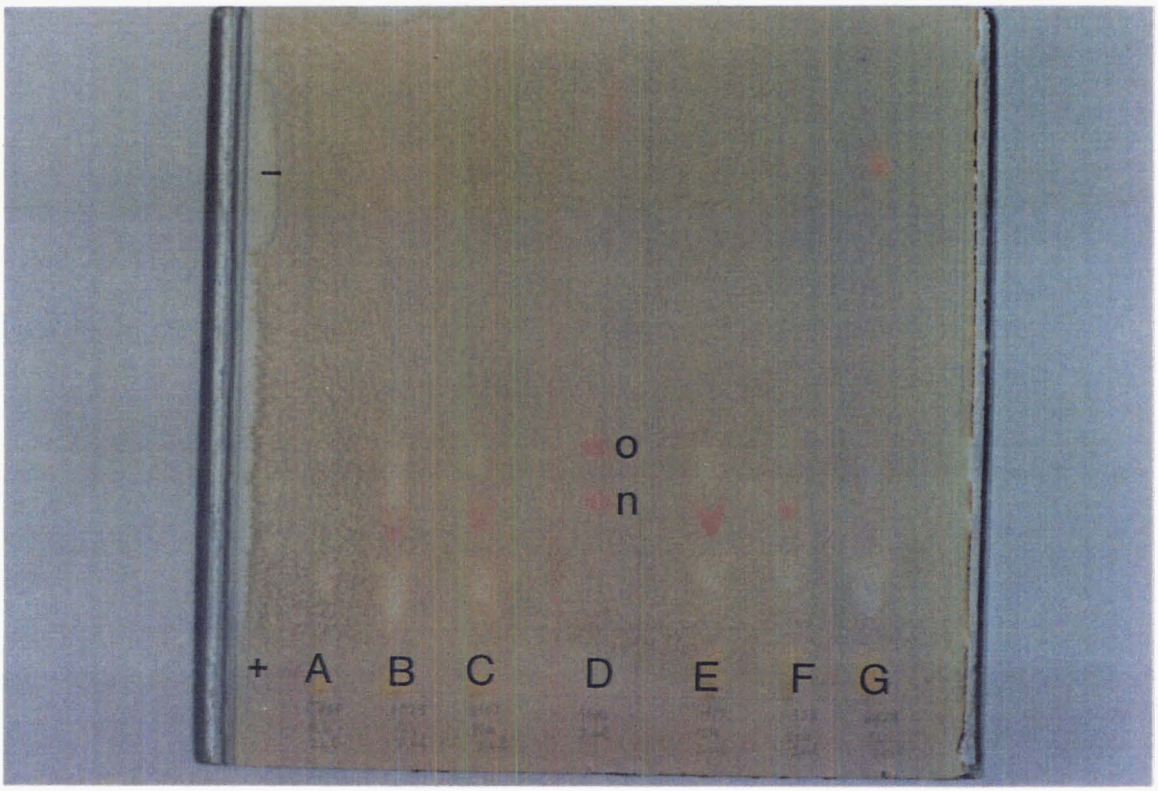
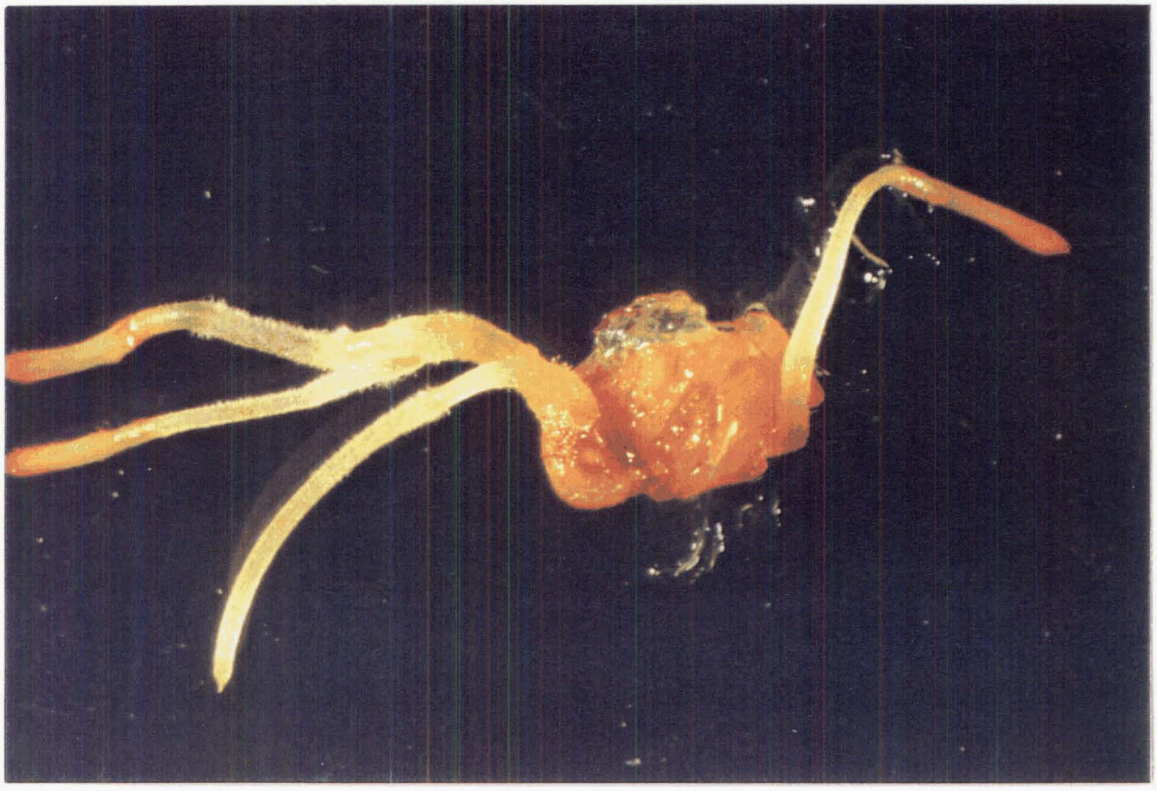
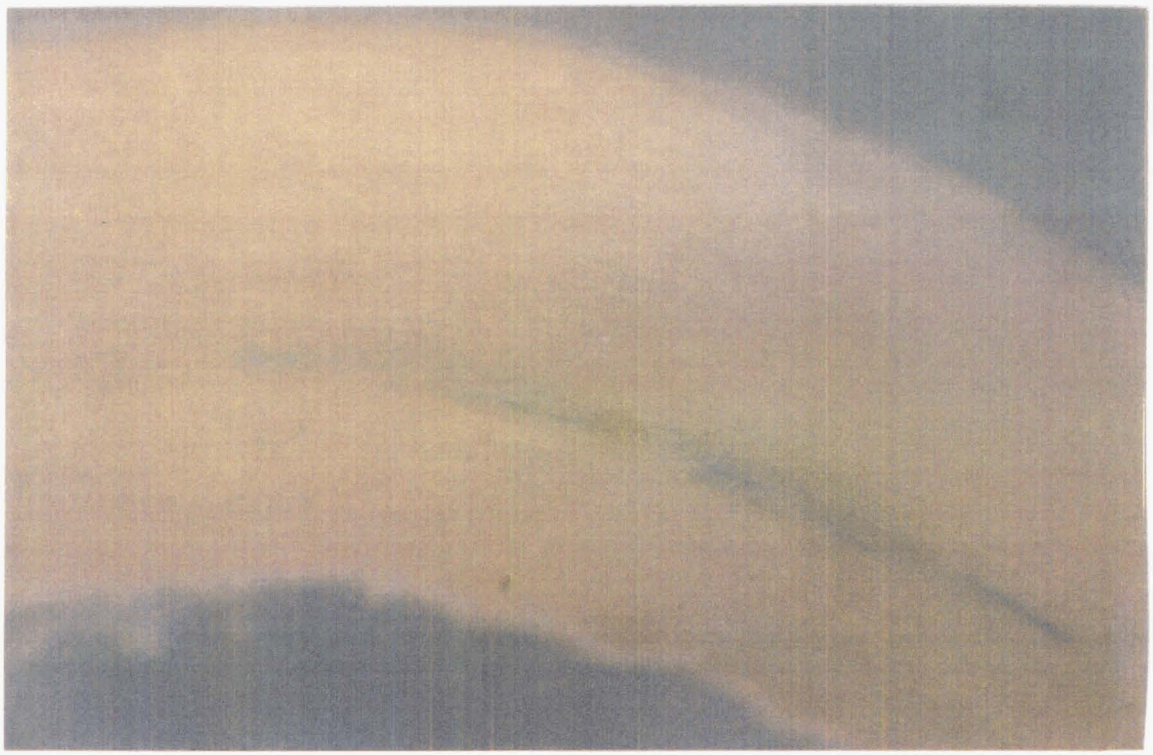

–
o
n
+ A B C D E F G

Plate 2.5 Roots produced by an excised *in vitro*-cultured tumour which has been in culture for eight weeks (x10 actual size). The tumour resulted from inoculation of a 'PLK' onion bulb with the strain A4T, harbouring pKIWI110.

Plate 2.6 GUS activity in the vascular region of a root produced by a tumour (x40 actual size). This tumour was the result of inoculations of 'PLK' bulb bases with the strain A4T, harbouring pKIWI110.





Chapter 3: Tissue and protoplast culture of *Allium cepa*

3.1. Abstract

Eight onion (*Allium cepa*) genotypes were screened in tissue culture for callus formation, regeneration of plantlets from callus and clonal multiplication by shoot proliferation. All of these genotypes could be clonally multiplied and four of them were regenerable from callus. A technique for plantlet multiplication, which uses longitudinally-bisected stems of 4-6 week-old *in vitro*-germinated onion seedlings as explants, was developed.

Onion ('Pukekohe Longkeeper', 'Southport White Globe', 'Japanese Saporo Yellow' and 'Hikeeper F1') protoplasts were isolated and cultured on a range of specialized media. These protoplasts often resynthesized cell walls and sometimes divided. However, only first cell divisions were regularly seen. After they had formed walls or divided, protoplasts remained alive and intact for up to six weeks. Culturing protoplasts in the same media solidified with 0.8% agarose, also resulted in cell wall formation and first cell divisions.

3.2. Introduction

The assumption that whole transformed plants can be regenerated from transformed protoplasts, cells and tissues is implied within all gene transfer techniques currently in use for plants. The inability of plants to be regenerated from transformed cells or tissues is usually the final barrier to the production of genetically transformed plants. Plant regeneration from tissue cultures is most commonly accomplished via

somatic embryogenesis or organogenesis. Prior to the development of an efficient *Agrobacterium*-mediated transformation system for onion cultivars, tissue culture systems must be established for the genotypes of interest. Important components of the tissue culture system include the chemical and physical environment, as well as the choice of plant genotype and the explant source (Conner and Meredith, 1989).

Methods currently available for the *in vitro* propagation of onion include:

1. shoot multiplication from axillary buds in meristem tip cultures (Havel and Novak, 1985),
2. direct formation of adventitious shoots on explants removed from basal plates (Hussey and Falavigna, 1980) or from flower heads (unripe umbels) (Matsubara and Hihara, 1977; Dunstan and Short, 1979) and
3. indirect formation of adventitious shoots and/or somatic embryos on callus tissues established from the proliferating cells of explants (e.g. basal plates, leaves, immature inflorescences, segments of roots, ovules, anthers etc.) (see Table 3.1).

The first two methods generally produce plants which are genetically unaltered from the explants used (Novak, 1990). The third method, which involves a callusing phase, may result in regenerated plants which have altered genotypes due to somaclonal variation in culture (Larkin and Scowcroft, 1981; Novak, 1990).

The literature provides considerable information on regeneration of *Allium cepa* plants from callus, derived from a range of explants and from single cells (Table 3.1). To achieve the *in vitro* regeneration of onions from undifferentiated callus, a protocol for the induction and maintenance of callus is needed. Dunstan and Short (1977) have modified the basal B5 medium of Gamborg (1968) by increasing the levels of ammonium, phosphate and nitrate salts, and with the addition of 2,4-D, obtained rapidly growing, friable onion callus. This callus was initiated from stem tissue excised from sets (bulblets often used for onion propagation). Shoots were regenerated from the callus following transfer to a medium containing the cytokinin,

Table 3.1 A summary of explant types and starting tissues used in tissue culture of *Allium cepa* and other *Allium* species.

	Explant type			
	Callus	Regeneration	Shoot proliferation	Haploid plants
Allium cepa (onion)	set[1] bulb[1] radicle[1] aerial bulbs (topsets)[2] seedling shoot meristem tips [12] seedling stem [16] shoot tips [8,12] umbels [8]	callus (via organogenesis)[1] callus (via somatic embryogenesis)[8,10,12] cell suspension cultures (via somatic embryogenesis)[10]	twin scales[3] inner scales[4] immature flower buds[5] axillary buds[7] shoot tips[8] umbels[8] aerial bulbs (topsets)[8] somatic embryos[12]	unpollinated ovules[9] unpollinated ovaries[19]
A. sativum (garlic)	young leaves[13] shoot meristems[14] storage leaves[17]	Callus (via organogenesis and somatic embryogenesis)[11,17,18]	shoot meristems[15]	
A. fistulosum (bunching onion)	radicle [6]	callus (via somatic embryogenesis)[6,8]		
A. altaicum	umbels [8]	callus (via somatic embryogenesis)[8]		
A. galanthum	umbels [8] topsets [8]	callus (via somatic embryogenesis)[8]	umbels, topsets (= aerial bulbs)[8]	
A. roylei	shoot tips [8]	callus (via somatic embryogenesis)[8]	shoot tips[8]	
A. chinense (scallion)	shoot meristems [14]			
A. tuberosum (Chinese chives)	seedling stem [16]	callus (via organogenesis and somatic embryogenesis)[16]		

1. Dunstan and Short (1978)
2. Fridborg (1971)
3. Hussey and Falavigna (1980)
4. Yoo *et al.* (1990)
5. Pike and Yoo (1990)
6. Shahin and Kaneko (1986)
7. Hussey (1980)
8. Phillips and Hubstenberger (1987)
9. Campion and Alloni (1990)
10. Phillips and Collins (1983)
11. Novak and Betlach (1981)
12. Phillips and Luteyn (1983)
13. Lu *et al.* (1982)
14. Oosawa *et al.* (1981)
15. Bhojwani (1980)
16. Dommisse (1993)
17. Zhou *et al.* (1980)
18. Novak (1980)
19. Muren (1989)

6-(3-methyl-2-buten-1-ylamino) purine (2iP), and the auxin, naphthaleneacetic acid (NAA) (Dunstan & Short, 1978). Phillips and Luteyn (1983) used the auxin-like PGR 4-amino-3,5,6-trichloropicolinic acid (picloram) in combination with the cytokinin 6-benzylaminopurine (BA) to induce and maintain friable, healthy onion callus from seedling shoot meristem tips, and to stimulate regeneration of plants from this callus. They also showed that picloram was superior to 2,4-D for continued maintenance and friability of callus, and subsequent regeneration of plants. Work has also been done on regeneration of garlic (*Allium sativum*) and other *Allium* species and this information is summarized in Table 3.1.

A range of explant types were used in *Agrobacterium* transformation experiments described in this thesis (section 5.3.2). As regeneration of plants from these explants would be necessary for the production of transformed plants, existing protocols were examined and new ones established for the regeneration and clonal propagation of nine onion cultivars. Attempts to find a suitable protocol for the regeneration of onion plants from protoplasts are also described in this chapter. Transformation of protoplasts either via *Agrobacterium* or by direct DNA uptake requires such a protocol to have been established in order for transformed plants to be recovered.

3.3. Materials and Methods

3.3.1 Onion callusing

To determine the optimal concentrations of picloram and BA needed for callus production from part or parts of *Allium cepa* 'Pukekohe Longkeeper' ('PLK') seedlings, a small range of picloram-BA combinations (Table 3.2) was set up on BDS basal medium (Dunstan and Short, 1977), pH 5.5, containing 3% sucrose and solidified with 0.7% agar (Davis, Bacteriological). Seeds were surface-sterilized in a 30% v/v solution of commercial bleach (5.0% w/w sodium hypochlorite) with a drop of detergent for 30-45 mins. They were then rinsed at least three times in

Table 3.2 Responses of excised 'Pukekohe Longkeeper' seedling stems to various BA-picloram combinations, on which they were cultured in the dark for 12 weeks. Concentrations used were based on those recommended by Phillips and Luteyn (1983). Where callus resulted, the mean weight of callus produced per explant is given in brackets.

Picloram concentration (mgl^{-1})	BA concentration (mgl^{-1})		
	0	1.5	2.0
0	Shoots	Roots (long)	Roots (shorter)
0.75	Callus (mean = 0.825 g), Roots	Callus (mean = 0.715 g), Shoots	Callus (mean = 0.847 g), Roots
1.5	Callus (mean = 0.988 g)	Callus (mean = 0.446 g)	Callus (mean = 0.943 g), Roots

sterile water and plated on the picloram-BA media. They were cultured in the dark at 25°C for 12 weeks. Following the determination of the PGR concentration which optimally induced callus growth, sterilized seeds of *A. cepa* cvs 'PLK', 'Early Longkeeper' ('ELK'), 'Southport White Globe' ('SWG'), 'Hikeeper', 'Odorless', 'Violet de Galmi', 'Dorata di Bologna' and 'Japanese Saporo Yellow' ('JSY') were placed on this callusing medium in the dark at 25°C. Callus was induced from seedlings without subculture or transfer to another medium. This medium was also used for callus maintenance.

3.3.2 Regeneration experiments

The callus used in regeneration experiments was derived from culture on BDS supplemented with 1.5 mgl^{-1} picloram.

3.3.2.a *Regeneration on BA and picloram*

Regeneration experiments were set up on various combinations of picloram and BA with a wider range of concentrations than had been used for callus induction experiments. Callus pieces (5-8 mm in diameter) were cultured in the light (cool white fluorescent tubes, 30 $\mu E/m^2/sec$) under 16 h days, 8 h nights for 12 weeks. The callus used had been cultured for three months since initiation.

3.3.2.b *Regeneration on thidiazuron and picloram*

Regeneration experiments were carried out using thidiazuron (N-Phenyl-N^1-1,2,3-thiadiazol-5-yl urea) (TDZ) as the sole PGR added to BDS basal medium (see Table 3.3 for concentrations), or with TDZ and picloram. TDZ was dissolved in DMSO (10 mg ml^{-1}) and added after autoclaving, when media had cooled to about 37°C. Nine pieces of callus (5-8 mm in diameter) were placed on each treatment and cultured in the light (cool white fluorescent tubes, 30 $\mu E/m^2/sec$) for ten weeks, after which they were assessed.

Table 3.3 Responses of 'Pukekohe Longkeeper' callus to a range of thidiazuron concentrations. Four-week-old callus was cultured in the light for 10 weeks. Total numbers of roots, shoots, green buds and root-like structures are given in brackets.

Percentage of calli[1] forming:	Thidiazuron (TDZ) concentration (mgl^{-1})						
	0	0.2	0.5	1	2	4	8
Roots	89% (187)	89% (42)	78% (30)	67% (21)	67% (40)	89% (25)	45% (11)
Shoots	0%	0%	0%	0%	11% (13)	11% (16)	0%
Green buds	0%	56% (9)	56% (27)	67% (22)	78% (30)	78% (23)	67% (18)
Root-like structures	0%	0%	0%	11% (5)	0%	0%	0%

[1] Nine pieces of callus were plated on each different medium.

3.3.3 Cell suspension cultures

Most suspension cultures are obtained by transfer of friable callus lumps to agitated liquid medium of the same composition as that used for callus growth (Dixon, 1985). Friable, undifferentiated callus cultures of 'PLK', 'ELK' and 'SWG' were gently crushed with the back of a wide scalpel blade and placed in 50 ml sterile flasks (125 ml Erlenmeyer) containing liquid BDS supplemented with 1.5 mgl^{-1} picloram, as was determined to be optimal for onion callus growth (section 3.4.1), and in LS (Linsmaier and Skoog, 1965) medium containing 1 mgl^{-1} 2,4-D, a medium used for initiation and maintenance of *Brassica* cell suspension cultures (pers. comm. Dr Mary Christey). Flasks were stoppered with sterile cotton bungs, capped with aluminium foil and placed on an orbital shaker (Chiltern Scientific, model SS70) which rotated at 160-180 rpm. Single cells did not usually come away from starting callus to form uniform suspensions. Subsequently, fresh suspension cultures were started by filtering cell suspension inocula which contained clumps through a sterile, 100 μm pore size nylon filter (Uremesh).

3.3.4 Production of axillary and adventitious shoots from basal plate explants

3.3.4.a *Twin scales from onion bulbs*

Most of the bulb scales and roots of mature, field-grown 'PLK' and 'ELK' bulbs were peeled off and discarded. Corresponding basal plate tissues of the bulbs were also removed, leaving only the main shoot surrounded by the two innermost scales. This dissected bulb was cut transversely at its widest point and the lower half cut into eight equal sectors. From each sector a 'twin scale' was cut, approximately 10 mm high and 3-5 mm wide, joined at the base by a small piece of basal plate tissue approximately 1-3 mm high (Plate 3.1). The excised twin scales were surface-sterilized in a 25% v/v solution of commercial bleach (5% w/w sodium hypochlorite) by stirring for 30-40 mins. These were then washed at least three times in sterile distilled water and placed on media.

3.3.4.b *Split in vitro shoots*

In vitro shoots derived from twin scales were used as secondary explants after being split longitudinally. Shoots 3-5 mm in diameter were trimmed to a height of 10 mm and a thin slice of approximately 0.5 mm was cut from the base to remove old basal plate tissue. A longitudinal cut was made so as to divide the shoot into semicylindrical halves while destroying the main shoot apex. These explants were placed on a medium used for onion shoot proliferation (Hussey and Falavigna, 1980), of which the concentrations of NAA and BA were adjusted to 0.12 mgl^{-1} and 2 mgl^{-1} respectively, to suit growth and maintenance of *in vitro* 'PLK' shoot cultures. Initially, 4 mgl^{-1} of BA had been used, but after vitrification was occasionally observed in leaf tissue, the concentration of BA was halved, and the concentration of sucrose reduced from 3% to 2.5% (see section 4.5.2.d for discussion on vitrification).

3.3.4.c *Production of roots from shoot cultures*

To stimulate the induction of roots from basal plates of shoot cultures, shoots were transferred to the same medium, but with twice the amount of NAA and no BA. This medium modification was based on a general recommendation for rooting of shoots, made by Bhojwani and Razdan (1983).

3.3.5 Shoot production from seedling stems

Onion ('PLK', 'ELK' and 'SWG') seeds were surface-sterilized as described in section 3.3.1. and placed on 1/2x BDS macro-, micro- and iron salts (Dunstan and Short, 1977), which contained no sucrose or PGRs, and was solidified with 0.7% agar. They were germinated in the light (cool white fluorescent tubes, 30 μEinsteins m^{-2} sec^{-1}, 16h light, 8h dark) at 20°C. Seedlings were grown until the stem area of the seedling (Fig 3.1) which would later become the bulb, had swollen. At this stage the stem, which contains the apical shoot meristem, was excised by cutting

about 3 mm on either side of it. Regeneration experiments using these explants were carried out on BDS containing several combinations of picloram and BA (Table 3.4). In a subsequent experiment, excised seedling stems were bisected longitudinally, so that the apical meristem would be destroyed. Bisected stems were placed on the same media as the unbisected stems and cultured in the light (cool white fluorescent tubes, 30 μE m^{-2} sec^{-1}, 16h light, 8h dark) at 25°C for 10 weeks.

3.3.6 Isolation and culture of protoplasts

3.3.6.a *Isolation*

Protoplasts were isolated from hypocotyl and leaf tissue of *in vitro*-germinated seedlings, 15-30 days following germination, and from *in vitro* shoot cultures of 'PLK', ' SWG' and 'JSY'. They were also isolated from callus cultures of 'PLK', 'Hikeeper' and 'JSY', each of which had been growing on BDS containing 1.5 mgl^{-1} picloram. All tissues from which protoplasts were to be isolated were placed into a sterile pre-plasmolysis solution (0.6 M sorbitol, 3 mM $CaCl_2$, 3 mM MES), 3-6 hours prior to incubation with enzymes. As this pre-plasmolysis step had no noticeable effect on the yield and viability of the isolated protoplasts, when compared with that of protoplasts isolated from unpre-plasmolysed tissue, it was subsequently omitted from the protocol.

Seedling, shoot and callus tissues were incubated overnight in enzyme mixture (0.6M sorbitol, 3 mM $CaCl_2$ and 3 mM MES, with 1% cellulase 'Onozuka R-10' and 0.25% macerozyme 'R-10'). Both enzymes were obtained from Yakult Biochem, Nishinomiya, Japan. *In vitro*-grown leaves were cut into approximately 5 mm sections and placed in sterile glass 5 cm petrie dishes which contained 3 ml of the filter-sterilized enzyme mixture. Dishes were sealed with parafilm and incubated in the dark overnight at 25°C. The leaf-enzyme mixture was not agitated, as previous experiments had shown that agitation resulted in up to 50% more cell membrane breakage (data not shown). The next morning the leaf-enzyme mixture was gently swirled for 2-3 minutes to release protoplasts from cell wall material. Crushed callus

Table 3.4 'Pukekohe Longkeeper' bisected seedling stem explants on various BA/Picloram combinations. The basal medium used was BDS.

Pic (mgl^{-1})	BA (mgl^{-1})					
	0	0.25	0.50	0.75	1.00	1.50
0	several shoots several roots	shoots	shoots, roots	bulbil, roots, shoots	shoots, roots	shoots
0.25	white callus	yellow, nodular callus, shoots	rooty callus, shoots	yellow, nodular callus, shoots	yellow callus, shoots	callus, roots, shoots
0.50	nodular callus	yellow, nodular callus, shoots	yellow, nodular callus, shoots	yellow, nodular callus, shoots	nodular, white-green callus, shoots	yellow, nodular callus, shoots
0.75	callus, shoots	yellow-white callus, roots, shoots	callus	yellow callus, shoots	callus, shoots	little response
1.00	yellow, nodular callus, shoots	yellow, nodular callus, shoots	yellow, rooty callus	yellow callus, shoot	yellow callus, green- white callus shoots rooty callus	callus, shoots
1.50	callus, shoots	callus	large amounts of yellow, nodular callus (rooty), shoots	white callus, green callus, shoots	callus shoots	callus, shoots, roots
2.00	yellow callus, shoots roots	hard, yellow callus, shoots	callus	yellow, nodular callus, shoot	yellow callus	yellow, nodular callus (rooty), shoots

tissue was digested using the same enzyme mixture, but with slow agitation (50 rpm) on an orbital shaker (Mistral Multi-Mixer, Lab-line Instruments Inc). Leaf and callus protoplasts were separated from undigested cells, cell wall material and other debris by successive filtration through two sterile nylon filters (Uremesh), with pore sizes of 100 μm and 51 μm.

3.3.6.b *Washing*

After filtering, protoplasts were collected and suspended in 3 ml of washing medium. This medium consisted of V-KM medium (Bokelmann and Roest, 1983), containing 0.32M NaCl instead of glucose. Protoplasts were then collected by centrifugation (70g, 3 min). Following removal of the supernatant, 6 ml of 0.6M sucrose was added to the protoplast pellet, and viable protoplasts were separated from dead ones and other cellular debris by centrifugation (70g, 10 min). Viable protoplasts, which floated to the top of the sucrose solution, were pipetted off and resuspended in washing medium. They were then washed twice, and yields were determined by a cell count with a haemocytometer. The percentage of viable protoplasts isolated was estimated from small aliquots of protoplasts that were stained with fluorescein diacetate, and viewed with fluorescence microscopy (Larkin, 1976). Evan's Blue stain, which penetrates only those cells whose membranes are disrupted, was used to confirm the percentage of viable protoplasts (Plate 3.2).

3.3.6.c *Culture*

Washed protoplasts were resuspended in appropriate amounts of liquid or agarose culture media to bring the final density to approximately $2x10^5$ cells per ml. In some cases this density could not be achieved in sufficient volume, as fewer protoplasts had been isolated. Consequently, plating densities as low as $2x10^4$ cells per ml were recorded. A range of media types, culture vessels and PGR combinations was used to determine optimal culture conditions for protoplasts. The basal media used included 8p (Kao and Michayluk, 1975), V-KM (Bokelmann and Roest, 1983), MS, as modified by Wang *et al.* (1986) and BDS (Dunstan and Short, 1977). Details of

these media are given in Appendix I. To make 'conditioned media', filtered media from one-week-old onion and *Nicotiana plumbaginifolia* cell suspension cultures were added to protoplast media in varying ratios. In addition to this, thin layers of cell suspension cultures were put into the wells of multiwell plates (Nunclon, Denmark) adjacent to those containing protoplasts, or into the outer compartment of a culture dish with protoplasts cultured in the centre well (Falcon Labware, USA). A third method involved embedding cell suspension cultures in a thin layer of low-melting point agarose in 5 cm petri dishes (Nunclon, Denmark), above which protoplasts in liquid media were cultured.

A range of low PGR concentrations was added to the media. Concentrations originally specified for the four basal media were also trialled. Picloram and BA were generally used, as previous experiments had established their superiority, as compared to NAA and zeatin, or IAA and kinetin, in stimulating cell division and cell wall formation (data not shown). Some media were also solidified with 0.8% agarose (low gelling temp., Sigma Chemical Co.), to which protoplasts were added immediately prior to pouring into dishes. Protoplasts embedded in agarose media were cultured in thin layers, approximately 2 mm thick, in 5 cm Nunclon petri dishes.

Two to three weeks after liquid protoplast cultures were established, the osmoticum in the medium was 'diluted' down to one half of its initial concentration. This was done by the addition of 8p, V-KM, MS or BDS, lacking osmoticum, i.e. no mannitol in the case of V-KM, MS and BDS, or reduced glucose in the 8p medium in which glucose acts as both the osmoticum and the carbon source.

3.4. Results

3.4.1 Callus production

PGR concentrations which stimulated optimum callus growth from onion seedling

stems on BDS basal medium were determined. Callus was produced by 'PLK' seedling stems on all media containing picloram (Table 3.2). The highest weight of undifferentiated callus was produced from the stem region, i.e. the area at the junction of the hypocotyl (cotyledon) and the radicle (primary root) (Fig 3.1), of 'PLK' seedlings on 1.5 mgl^{-1} picloram and no BA (Table 3.2). Seedling stems of other cultivars surveyed produced similar amounts of undifferentiated callus on BDS containing 1.5 mgl^{-1} picloram (Table 3.5). Occasional shoots were produced from callus of all cultivars, while on this medium. These shoots were only seen 3-6 months after callus induction and were more commonly seen on dark- than light-grown callus.

3.4.2 Regeneration from callus

In the first regeneration experiments set up on combinations of picloram and BA, shoots were most consistently produced from onion callus on 1.6 mgl^{-1} BA and 0.4 mgl^{-1} picloram. Those cultivars producing callus from which plants could be regenerated are listed in Table 3.5. Shoots could only be consistently regenerated from callus less than six months old. Most of this callus was cream in colour and nodular in appearance. Callus older than six months readily produced roots when transferred to regeneration media or to PGR-free media. Differences were noted between different callus lines of the one cultivar, some regenerating more readily than others, but no specific data were collected on the regenerative capacities of different callus lines.

When TDZ was used as the sole PGR, regeneration of shoots from callus occurred on 2 and 4 mgl^{-1} TDZ (Table 3.3). Since shoot production occurred at low frequencies, interactions of PGRs and light/dark conditions were investigated in more detail. Calli were cultured in the light on combinations of TDZ and picloram. Eight weeks later they were split in two and one of each treatment was placed in both dark and light conditions. Four weeks later, shoots were visible on two of the light-grown treatments (1.5 and 3 mgl^{-1} TDZ) with green buds and roots being visible on a range of light treatments (Plate 3.3). No shoots were produced from calli

Table 3.5 Responses of nine onion genotypes on specific tissue culture media.

Onion genotype (cv)	Callus production (seedling stem)[1]	Shoot regeneration from callus[2]	Production of axillary and adventitious shoots[3]	Root formation from shoots in culture[4]
PLK	+	+	+	+
ELK	+	+	+	+
SWG	+	-	+	+
Hikeeper	+	+	+	+
Odorless	+	-	+	+
Violet de Galmi	+	+	+	+
Dorata di Bologna	+	+	+	+
CMS[5] (cv unknown)	-	-	+	+
Japanese Saporo Yellow	+	-	+	+

[1] Medium defined in section 3.4.1
[2] Medium defined in section 3.4.2.
[3] Medium defined in section 3.3.4.b
[4] Medium defined in section 3.3.4.c
[5] CMS = cytoplasmic male sterile line. Only bulbs of this line were available, so callus could not be induced from seedling stems and therefore regeneration from callus was also not carried out.

cultured in the dark.

3.4.3 Production of axillary and adventitious shoots from basal plate explants

Provided that shoots were not vitrified, an 8-fold increase in shoot number was observed every 6-8 weeks, i.e. on average, eight shoots were produced from each twin scale explant. Roots were occasionally produced from shoots on the shoot proliferation medium, but were produced more frequently (on 90% of the shoots) on the root induction medium.

3.4.4 *In vitro* responses of seedling stem explants

Longitudinally-bisected 'PLK', 'ELK' and 'SWG' seedling stem explants responded in a variety of ways to the range of concentrations and combinations of BA and picloram used. Responses of these explants included callusing, shoot production and root production, some explants producing callus, shoots and roots. The responses of 'PLK' explants are presented in Table 3.4. Bisected seedling stems produced many shoots and roots on the hormone-free BDS medium (Plate 3.4).

3.4.5 Cell suspension culture

Even when the most friable callus available was used to initiate cell suspension cultures, only a small proportion of cells dissociated from callus. These cells eventually formed small clumps rather than producing a homogeneous cell suspension. The density of single cells in suspension culture was usually 1-3 x 10^4 cells per ml. Modifications to basal media or to PGR concentrations did not result in increased cell densities. When sieved suspensions were used as starting inocula, the resulting cell suspensions showed poor growth and division.

3.4.6 Protoplast culture

During incubation of leaf and callus tissue in the enzyme mixture, the middle lamella

appeared to be digested first, resulting in the release of intact cells complete with walls. Hydrolysis of cell walls occurred after this (Plate 3.5). Shaking at slow speeds did not appear to affect the viability or quality of protoplasts isolated from callus. Most protoplasts isolated from seedlings and shoots floated to the surface of the sucrose solution after centrifugation, but callus protoplasts, which had smaller vacuoles and more dense cytoplasm, did not generally float in the sucrose. The sucrose floatation step was consequently omitted from the protocol for callus protoplast isolation.

After two days of culture in a range of media, cell walls were seen to be re-forming on some of the protoplasts (Table 3.6). Protoplasts which had been plated in low melting-point agarose survived longer than those in liquid media of the same composition. Cell wall formation (Plate 3.6) and cell 'budding' (Plate 3.6) occurred frequently in most protoplast cultures, but true cell divisions were only occasionally observed (Plate 3.6, Plates 3.7a and 3.7b). Of the protoplasts which divided, some did so within four days of isolation. These included callus protoplasts derived from the cultivars 'Hikeeper' and 'PLK'. Cell wall formation and budding were also more common in 'Hikeeper' and 'PLK' callus protoplasts. In general, most cell divisions occurred 5-12 days after protoplast isolation. Cell walls re-formed more slowly on leaf protoplasts than on callus protoplasts.

Although the onion protoplast isolation and culture protocol of Wang *et al.* (1986) was closely followed, the results recorded by this group, i.e. regeneration of plantlets from protoplasts of the onion cultivar 'Yellow-skinned Onion', could not be replicated using protoplasts derived from onion cultivars 'PLK', 'Hikeeper' and 'JSY'. This was in spite of several media modifications, including various concentrations of different PGRs, and adjusted levels of sugars. It was not possible to obtain the seed of 'Yellow-skinned Onion' from seed companies contacted in Hong Kong. When cultured in the medium devised by Wang *et al.* (1986), onion protoplasts formed new cell walls, and sometimes divided, but no further cell divisions occurred and protoplasts eventually died. Those protoplasts which divided generally had dense cytoplasms and small vacuoles. Reducing the concentration of

Table 3.6 Responses of onion protoplasts cultured in three different basal media. Where no specific additions to the media are mentioned in brackets, the medium used was exactly as specified by the authors, as listed below.

Cultivar	Media on which cell wall formation occurred	Media on which cell division occurred	Media on which cell budding occurred
Seedlings 'PLK' 'SWG' 'JSY' 'Hikeeper'	V-KM (0.1, 0.2 mgl^{-1} picloram)	V-KM (0.1, 0.2 mgl^{-1} picloram)	V-KM (0.1, 0.2 mgl^{-1} picloram)
Shoot cultures 'PLK' 'SWG' 'JSY'	W-MS with V-KM vitamins V-KM (0, 0.1 mgl^{-1} picloram); 1/2 x salts W-MS (no PGRs)	W-MS with V-KM vitamins V-KM (0, 0.1 mgl^{-1} picloram); 1/2 x salts W-MS (no PGRs)	 1/2 x salts W-MS (no PGRs)
Callus 'PLK' 'Hikeeper' 'JSY' 'SWG'	W-MS, KM8p (0.8% agarose) W-MS, KM8p KM8p (0.8% agarose)	KM8p (0.8% agarose) KM8p KM8p (0.8% agarose)	W-MS; KM8p (0.8% agarose); KM8p (2 mgl^{-1} picloram) W-MS KM8p (0.8% agarose)

Media abbreviations:

V-KM = the macro elements of the V-47 medium (Binding, 1974) without NH_4NO_3, and the other nutrients of the KM8p medium (Kao and Michayluk, 1975). This medium was devised by Bokelmann and Roest (1983).

KM8p = a medium devised especially for growth of *Vicia hajastana* protoplasts at low densities in liquid media, by Kao and Michayluk (1975). The mineral salts used were modified from B5 (Gamborg *et al.*, 1968).

W-MS = The mineral salts of MS (Murashige and Skoog, 1962) medium, B5 organics and other components as devised by Wang *et al.*, (1986) for the culture of *Allium cepa* 'Yellow-skinned Onion' protoplasts.

mineral salts specified in the Wang *et al.* (1986) medium by half also resulted in first cell divisions, cell wall formation and budding of 'JSY' leaf protoplasts (Table 3.6).

3.5. Discussion

Protocols for callus initiation and proliferation, regeneration from callus, clonal propagation and protoplast isolation of selected cultivars of onions have been established during the course of this study.

3.5.1 Callus production

Compared to methods for the initiation and maintenance of onion callus described in the literature (Table 3.1), the method developed in this study appears to be simpler and less time-consuming. Given that seeds are easily surface-sterilized, and can be placed directly onto the callusing medium, and also that callus can be produced from the stems of seedlings within 3 weeks of germination, this technique enables the rapid production of callus. Explant surface-sterilization and dissection of the plant material no longer present a problem as onion seeds are relatively simple to sterilize, and no dissection of tissue is required.

3.5.2 Regeneration experiments

Shoots were only regenerated from onion callus with any degree of reproducibility if this callus was less than six months old. The loss of regenerative capacity by onion callus tissue after six months has also been reported by Dunstan and Short (1978), Davey *et al.* (1974) and Fridborg (1971). Phillips and Luteyn (1983) found that plants could be regenerated from 6-12 month old callus, but only on very specific media, and at low frequencies. Van der Valk *et al.* (1990) found that shoots could be regenerated from callus which was up to 13 months old, but this was only recorded for one of the six cultivars they studied. Other cultivars had lost their

regenerative capacity before this time. This inability of older callus to regenerate shoots or embryos is not confined to onions and has been seen in a range of other species (Lin and Griffin, 1992; Jain and Datta, 1992; Cheng and Smith, 1975; Smith and Street, 1974).

The occasional spontaneous regeneration of shoots from callus growing on callusing medium has not been recorded by other groups working on onion tissue culture. This regeneration on a medium which normally suppresses differentiation, suggests that the primordia responsible for the production of shoots must be 'strong'. Alternatively, shoots could have grown from pre-formed meristems that had never callused.

In this study, it was established that a dark period was not necessary for regeneration of shoots from callus. This is in agreement with the results of Phillips and Luteyn (1983) who also found that dark pre-treatments were not required for onion regeneration. However, Dunstan and Short (1978) and Swamy (1983) found the provision of a dark period to be critical for shoot formation from onion callus. It is likely that interactions between PGRs and light/dark affect the final outcome of onion regeneration experiments. Phillips and Luteyn (1983) observed the production of shoots and embryoids from callus, without a dark pre-treatment, when picloram and BA were used in regeneration media. However, Dunstan and Short (1978) and Swamy (1983) had used 2iP and NAA in regeneration media.

The ability of onion callus to readily regenerate roots regardless of age has also been recorded by other workers. Dunstan and Short (1978), Davey *et al.* (1974) and Fridborg (1971) all found that older onion callus maintained its ability to produce roots, even when shoots or plantlets could no longer be regenerated. The varying regeneration abilities of different callus lines of a single onion cultivar as seen in this thesis has also been observed by other workers. Phillips and Luteyn (1983) found that somatic embryos were produced at high frequencies only in some callus lines of 'Yellow Grano' and 'Yellow Sweet Spanish'. They also found the ability of callus tissue to produce green buds or shoots to be dependent on the line used.

Thidiazuron, a compound known to have cytokinin-like properties, has been used for regeneration of plantlets from callus of other plant species, e.g. apples (Dufor *et al.*, 1986) and *Rosmarinus officinalis* (Tawfik and Read, 1990). When used as the sole PGR, it stimulated regeneration of shoots from onion callus, but at low frequencies. More experimental work is needed to define which concentrations of TDZ are optimal for shoot regeneration.

3.5.3 Cell suspension cultures

One possible reason for suspension-cultured cells dividing slowly or not at all is that the density of cells in liquid media was too low. It is also possible that the suspension culture medium used (BDS with 1.5 mgl^{-1} picloram), although optimally suited to callus growth when solidified with agar, was not optimal for the growth and division of suspension-cultured cells.

To start or to subculture a cell suspension culture, a relatively large initial inoculum generally ensures that sufficient single cells and/or small clumps of cells are released into the medium to provide a sufficiently high cell density for subsequent growth. Although large amounts of apparently friable onion callus were used as inocula, this callus released only a small number of individual cells, or clumps of cells, when agitated, resulting in low cell densities. This problem may have been alleviated by the digestion of calli with a pectinase. This would have released a large number of individual cells for inocula. Onion cell suspension cultures may also have been induced to grow and divide by the addition of 'conditioned' media. To optimize the onion cell supension culture system, experiments with both enzymatic release of cells from calli, frequency of subculture, and the culture of low density suspensions in conditioned media, need to be carried out. Onion cell suspension cultures could not be used for planned purposes (e.g. feeder cells for protoplast culture) due to the problems encountered.

3.5.4 Production of axillary and adventitious shoots from basal plate explants

The technique and media (as modified in this project) of Hussey and Falavigna (1980) ensured that a regular supply of onion shoot cultures was available for use as starting tissue for protoplast isolations and *in vitro* injections with *Agrobacterium*. The rapid clonal multiplication of plantlets following initiation and subculture of shoot cultures will allow rare genotypes (e.g. single transgenic plants) to be clonally multiplied within a short period of time.

3.5.5 Shoot production from seedling stems

The technique of bisecting seedlings and plating them on PGR-free medium, which resulted in a quick shoot multiplication response, has not previously been reported. This rapid response is probably due to young seedling tissue being fast-growing. Advantages of this method over that of multiplying shoots from twin scales include:
(a) the sterility of *in vitro*-germinated seedlings as compared with field grown bulbs, which can be very difficult to sterilize for *in vitro* culture,
(b) the absence of any vitrified tissue in seedlings and
(c) the year-round provision of explants for co-cultivation with *Agrobacterium*.
As with the twin scaling method described in section 3.3.4, bisection of seedlings usually destroys the apical meristem. Therefore, after co-cultivation with *Agrobacterium* harbouring binary vectors with selectable marker genes, only transformed axillary meristem cells will produce healthy shoots on selective media. This will reduce the frequency of potential escapes arising from rapid elongation of pre-existing shoot meristems.

3.5.6 Protoplast isolation and culture

During the course of this work, it was observed that some of the media and conditions used induced first cell divisions, cell wall formation and cell budding. It is unclear as to why subsequent divisions were not seen, and why the cells would invariably die after having been alive for up to six weeks. Treatment of plant cells

with crude enzyme preparations that probably contained unidentified contaminating compounds (Evans and Bravo, 1983) may have had a deleterious effect on cell viability. Patnaik *et al.* (1981) found that plating efficiency of *Petunia parodii* leaf protoplasts could be increased significantly by using purified enzyme for protoplast isolation. High osmotic concentrations in early culture, and the accumulation of metabolic compounds in the culture media may also have adversely affected the viability of protoplasts. However, this does not explain why some protoplasts remained viable in culture for so long but did not divide.

It is possible that protoplasts which died early on in culture were producing substances, such as phenolics (Evans and Bravo, 1983), which, when released into the medium, might have inhibited those cells still alive, causing eventual death. Another factor which may have been responsible for the lack of subsequent cell divisions is the plating density of protoplasts. Although protoplasts were generally plated at a density of approximately 2 x 10^5 cells per ml, densities were sometimes lower due to poor release after digestion, or losses during the floatation step. The density of protoplasts was always halved when the osmoticum was diluted, but under ideal conditions protoplasts would have divided two or three times before this step, so that the dilution would only have restored the density of cells to its initial amount.

The lack of protoplast divisions could also be attributed to hormonal regimes, or to one or more other media ingredients not being present at the concentrations required to stimulate cell division. Von Arnold and Eriksson (1977) have proposed that concentrations of iron, zinc and ammonium salts are too high in some of the standard media for the culture of protoplasts of selected species. These standard media included MS, the mineral salts of which were used in the medium devised by Wang *et al.* (1986) for onion protoplast culture. The concentration of ammonium is considerably higher in MS medium than in any of the other three media used (Appendix I). This was why a range of basal media were used in this study, in which one or several of the macronutrients were present in different amounts or, in some cases, absent altogether. Van der Valk *et al.* (1990) isolated protoplasts from leaves of young onion seedlings and callus, and cultured these protoplasts on a range

of media including MS and V-KM. They observed no responses from seedling-derived protoplasts, and only protoplasts derived from the callus of a single cultivar ('Jumbo') formed new cell walls and showed budding. No true cell divisions were seen in the course of their work.

The observation that dividing onion protoplasts were almost inevitably those with dense cytoplasm agrees with those made for other species. Yamada *et al.* (1986) used rice suspension cultured cells which were round and rich in cytoplasm, as their source of cells from which to isolate protoplasts for regeneration, and Spangenberg *et al.*(1985) found that small *Brassica napus* protoplasts, which were rich in cytoplasm, entered division cycles earlier than large, highly-vacuolated protoplasts.

The reason why the results of Wang *et al.* (1986) were unable to be replicated, despite methods and materials being closely followed, is unclear, but a major factor is likely to be the use of a different cultivar. Different responses from protoplasts of different cultivars of the same species, have been recorded for a number of species, including rice (*Oryza sativa*) (Thompson *et al.*, 1986), cucumber (*Cucumis sativus* L.) (Jia *et al.*, 1986), *Petunia hybrida* (Frearson *et al.*, 1973) and *Arabidopsis thaliana* (Damm and Willmitzer, 1988).

The progress of the protoplast work presented in this thesis seems to fall somewhere between that of van der Valk *et al.* (1990) and Wang *et al.* (1986), the former group achieving only formation of cell walls, the latter regeneration of plantlets. Although in this study cell division was sporadic, and regeneration from protoplasts was not achieved, the establishment of a protocol for protoplast isolation does allow for the assay of transiently expressed genes following direct DNA uptake by protoplasts.

3.5.7 Further work

Although effective and novel tissue culture systems have been established for the cultivars studied, further work is required on the regeneration of callus, and ultimately plants, from protoplasts. Additionally, a more efficient system for

regeneration of plants from callus needs to be established for these cultivars, to increase the number of plants produced from each callus.

Fig 3.1 An onion seedling at the 'loop' stage, approximately 8-15 days after germination, with the stem region at the junction of the hypocotyl (cotyledon) and the radicle (primary root). This figure is reproduced from Jones and Mann (1963).

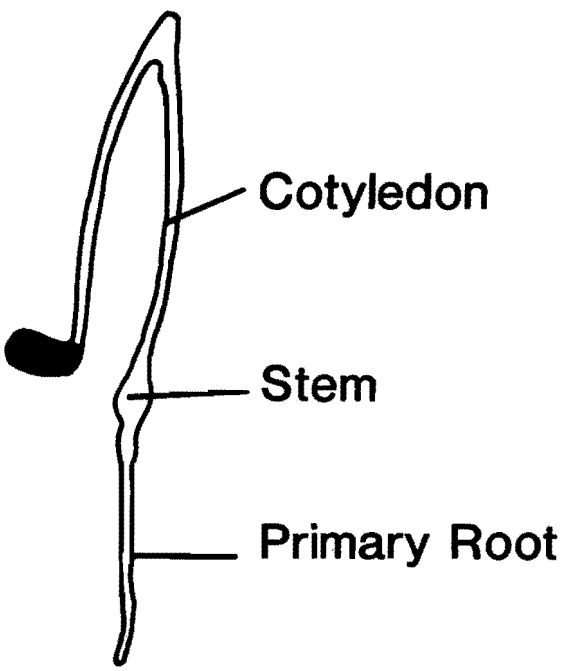
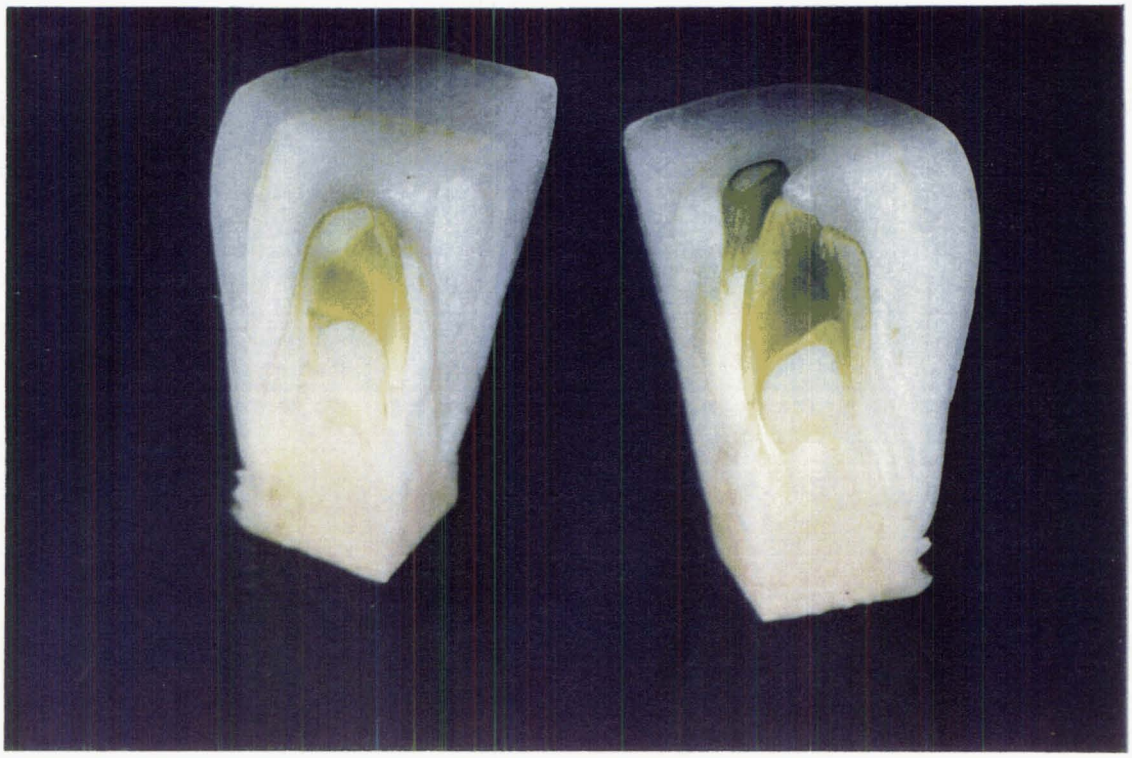

Cotyledon
Stem
Primary Root

Plate 3.1 Twin scales excised from a 'Pukekohe Longkeeper' bulb. Two bulb scales are still attached to the basal plate, and the shoot apex is longitudinally bisected.

Plate 3.2 Protoplasts isolated from *in vitro*-grown 'Pukekohe Longkeeper' leaf tissue and stained with Evan's blue. Only protoplasts with cell membrane damage (arrowed) are stained blue.



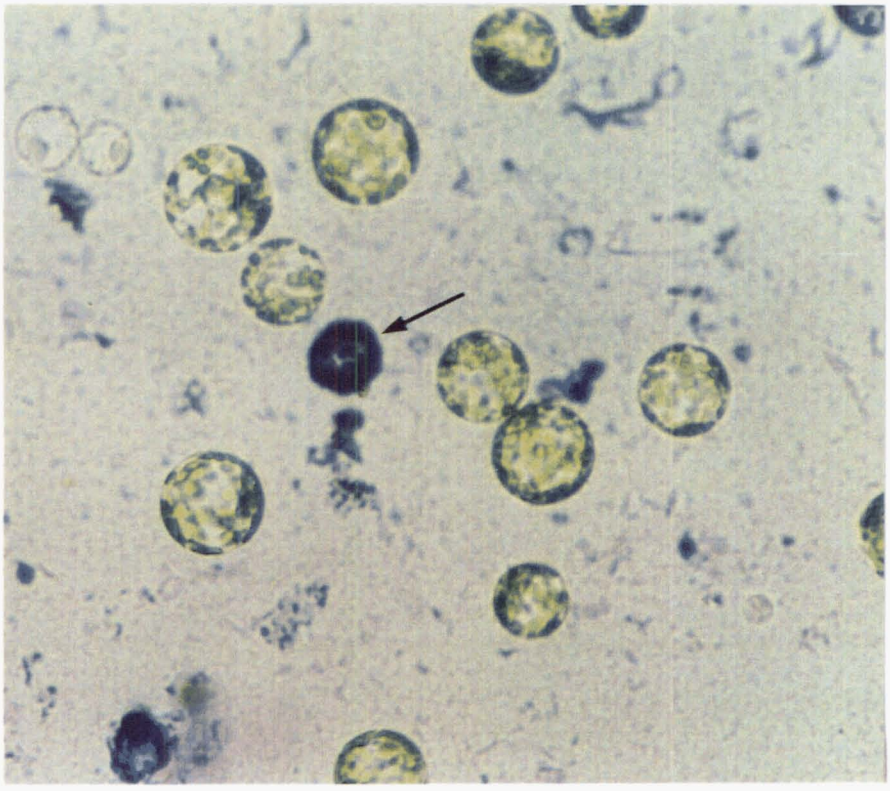
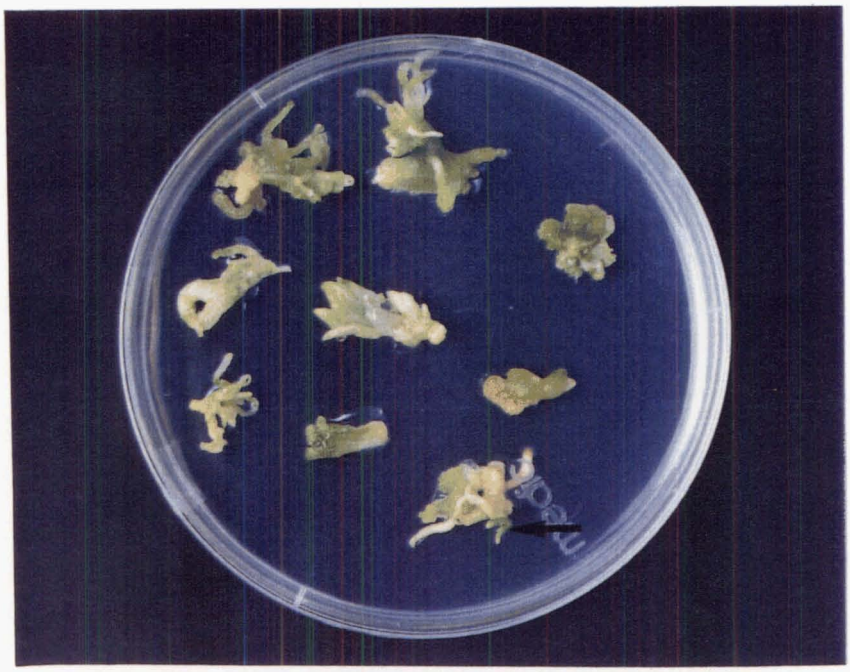


Plate 3.3 Regeneration of (a) shoots (arrowed) and (b) roots from 'Pukekohe Longkeeper' callus grown on media containing thidiazuron. Shoots were regenerated on 3 mgl^{-1} thidiazuron and roots on 0.2 mgl^{-1} thidiazuron. Regenerated shoots (c) are shown on shoot proliferation medium five days after transfer from the medium in (a).



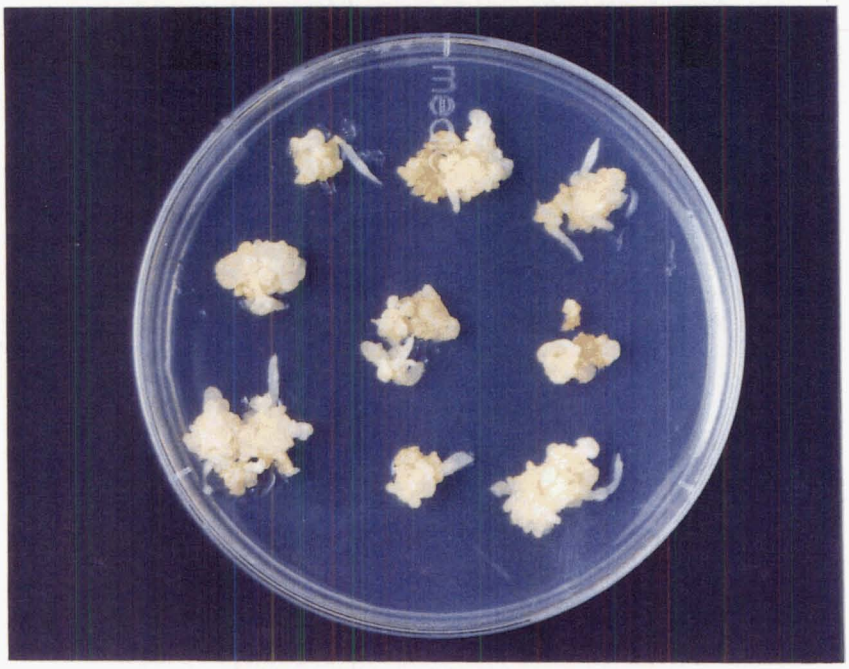
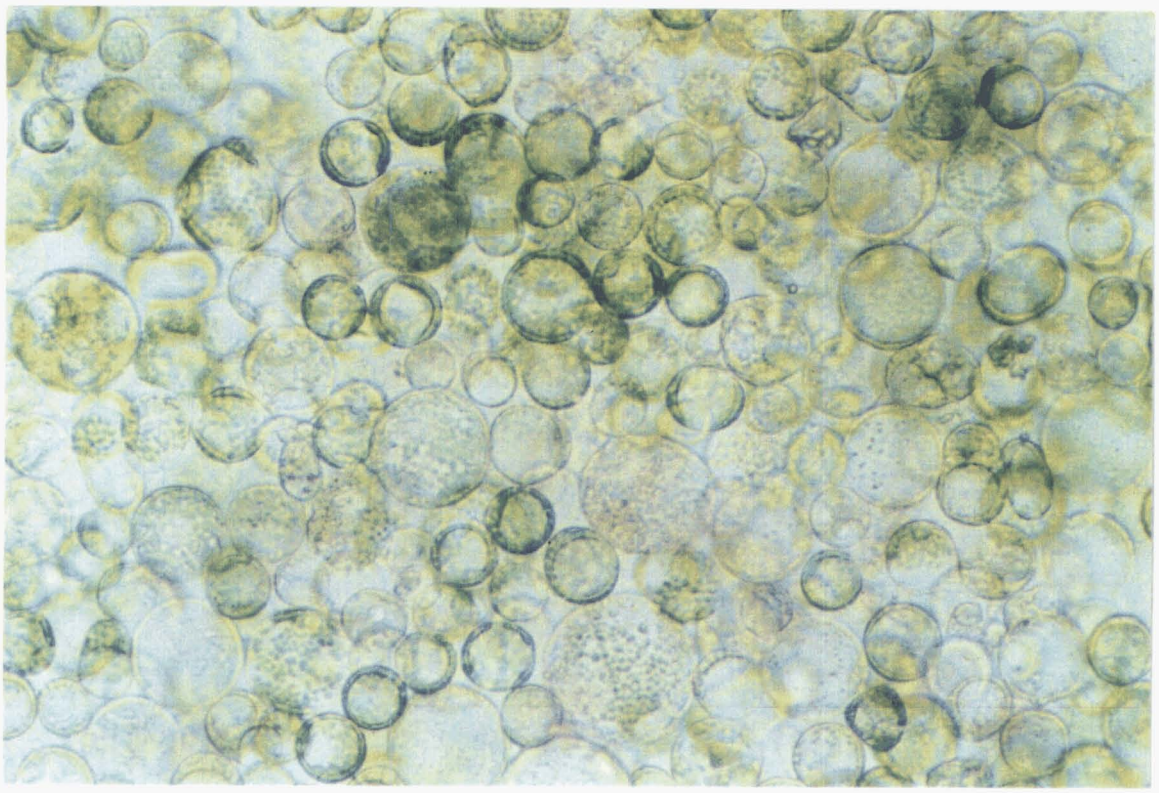


Plate 3.4 Shoots and roots produced from a bisected seedling stem on PGR-free BDS medium. Seedling stems have been in culture for seven weeks.

Plate 3.5 Freshly isolated onion leaf protoplasts

Plate 3.6 The first cell division of a protoplast (top) and formation of cell walls by 'budding' protoplasts (bottom). Protoplasts were isolated from *in vitro* shoot cultures of 'Pukekohe Longkeeper' and have been in culture for five days on V-KM medium with 2% sucrose, 0.5M mannitol, 2 mgl^{-1} 2,4-D and 0.5 mgl^{-1} BA.



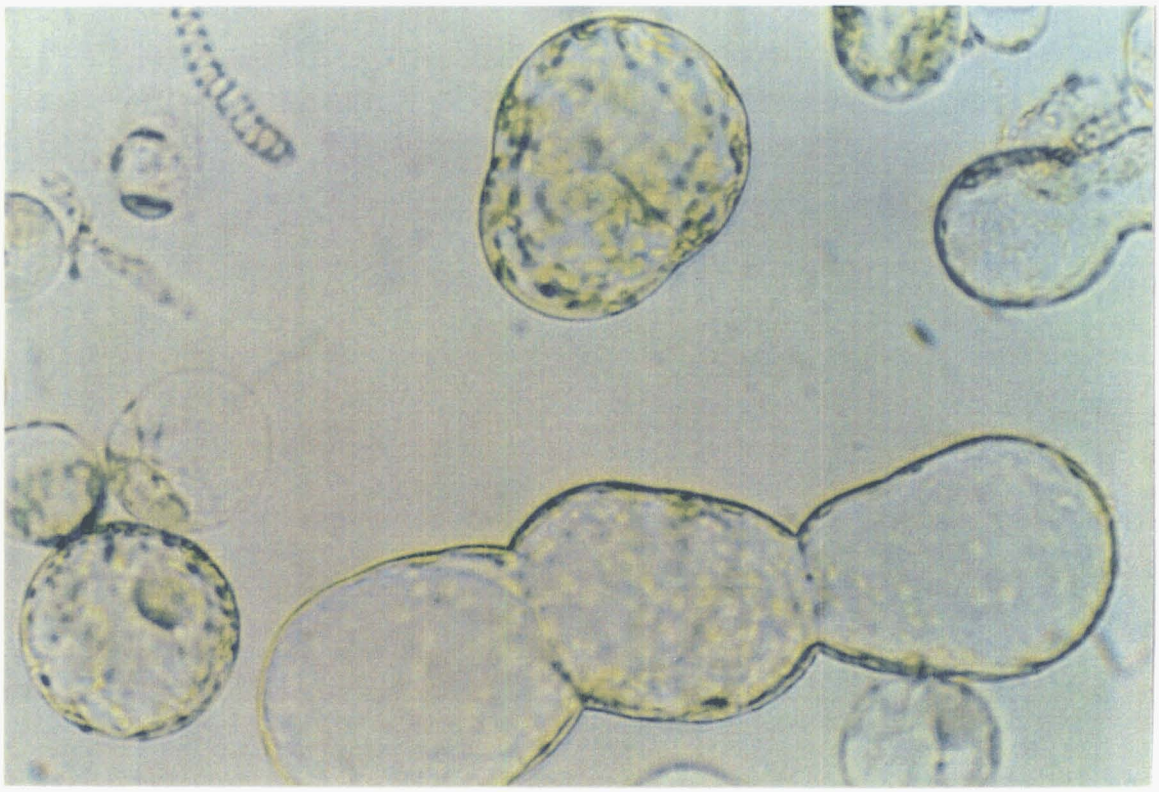
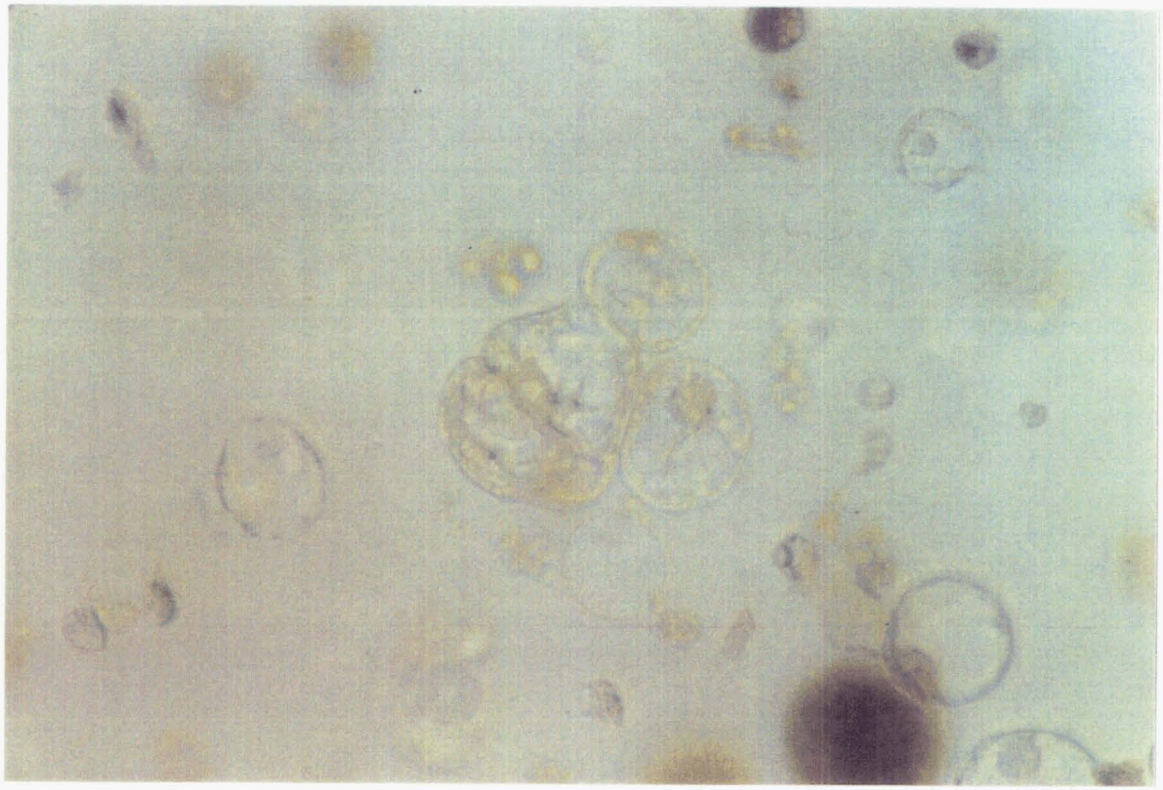


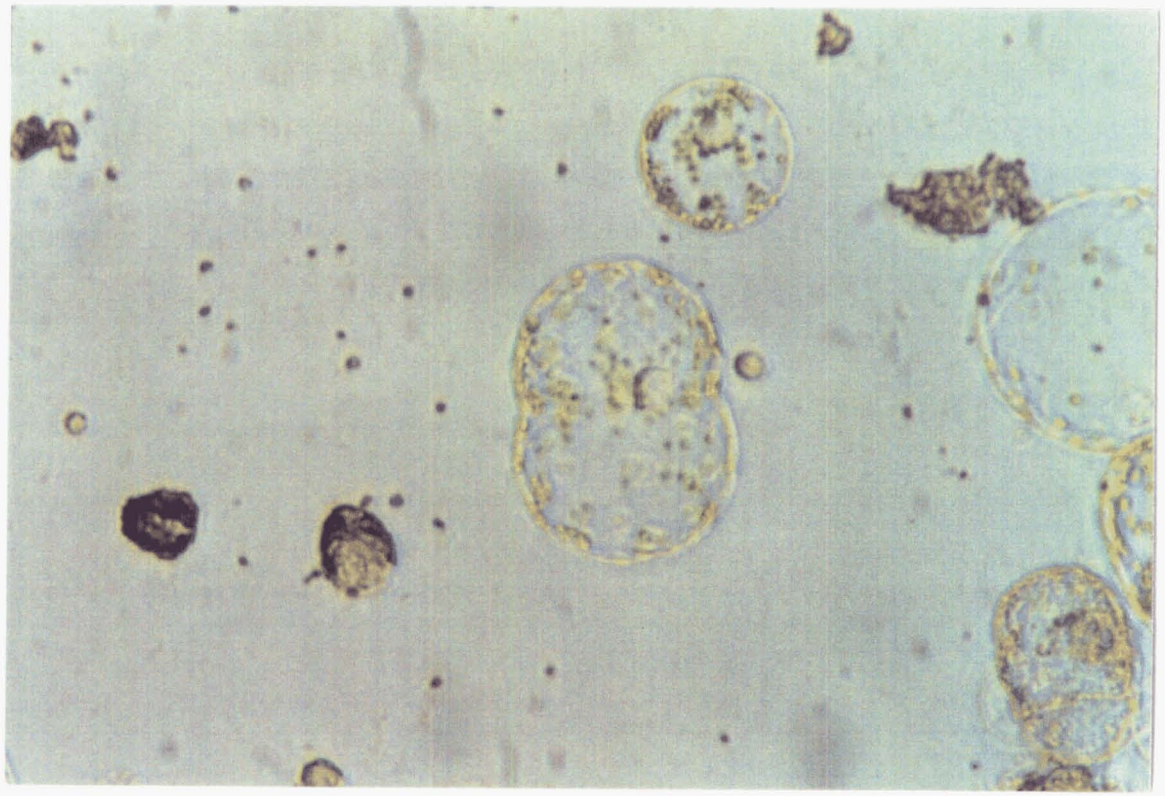
Plate 3.7 (a) The first cell division of a 'Pukekohe Longkeeper' callus protoplast after six days in culture. Protoplasts were cultured on 8p medium without PGRs and solidified with 0.8% agarose.

(b) The first cell division of a protoplast derived from 'Japanese Saporo Yellow' shoot cultures. Protoplasts were cultured on a PGR-free liquid W-MS medium with half the concentration of macro- and micro-salts.

a



b



Chapter 4: Responses of onion *in vitro* cultures to selective agents

4.1. Abstract

The antibiotics kanamycin, geneticin (G418) and hygromycin, and the herbicide chlorsulfuron were added to tissue culture media in a survey of responses of *in vitro*-cultured onion tissues to selective agents. Tissues surveyed included seeds and seedlings on germination and callusing media, established callus on callusing and regeneration media, and shoot cultures on shoot proliferation medium. All cultures were derived from the onion cultivars 'Pukekohe Longkeeper', 'Early Longkeeper' or 'Southport White Globe'. Hygromycin was shown to be the antibiotic most toxic to onion tissues, with toxicity effects being obvious in all tissues after 4-5 weeks of culture on concentrations as low as 20 mgl^{-1}. Kanamycin was the least toxic of the three antibiotics, as 'Pukekohe Longkeeper' callus tissue survived on concentrations of up to 750 mgl^{-1}, after being in culture for 12 weeks. By contrast, at concentrations of 30 mgl^{-1} or more, the kanamycin analogue G418 was toxic to most onion cultures. However, responses of tissues to G418 were slower than those to hygromycin and although most tissue types died after eight weeks on media supplemented with 30 mgl^{-1} G418, both young and established callus survived on this concentration for up to six months. Low concentrations (25-150 nM) of the herbicide chlorsulfuron were also shown to be toxic to onion seedlings and shoot cultures.

4.2. Introduction

Selectable marker genes are seen to be essential for the introduction of agronomically

important genes into crop plants via plant transformation. The use of antibiotics in plant tissue culture media has become widespread in recent years, as selection on antibiotics is included in the method of *Agrobacterium*-mediated transformation of most plants. In early *Agrobacterium* transformation experiments, opine synthesis genes from T-DNA were used as markers in the screening for transformed cells (Reynaerts *et al.*, 1988). Currently, cells containing a foreign gene insert are usually selected for by neighbouring antibiotic- or herbicide-resistance selectable marker genes. Only plant cells that contain and express the selectable marker gene will survive a selective pressure imposed in culture and plants regenerated from these surviving cells are likely to contain the selectable marker joined to the agronomic gene of interest.

In cell selection experiments, an occasional variant cell that can tolerate amounts of selective agent normally toxic to wild-type cells, may arise in culture (Binding *et al.*, 1970; Chaleff and Ray, 1984). Although it was originally thought that such variation was due just to mutant genotypes, the apparent high frequency and stable nature of many variants has since led to the postulation of other genetic or epigenetic mechanisms such as developmental (Binns, 1981) or somaclonal (Larkin and Scowcroft, 1981) variation. Such variants can survive selection in transformation experiments. Other cells or plants which can survive selection, despite the lack of genetic or phenotypic mechanism/s to cope with it, are known as 'escapes'. Depending on the stringency of selection, escapes may represent a large proportion of initially-selected cells or plants. In the context of transformation, escapes can be identified and eliminated after failure to survive a further cycle or cycles of screening or selection (Conner, 1986)

4.2.1 Types and examples of marker genes

Two main classes of selectable traits have been used in genetic modification of plants. These include selectable marker genes encoding proteins which confer resistance to antibiotics, and those whose products confer tolerance to herbicides. The most commonly used selectable marker is the gene from transposon 5 (Tn5) of

Escherichia coli K12, encoding aminoglycoside 3-phosphotransferase II [APH (3') II, Chemical Abstracts Registry number 58943-39-8]. This enzyme, more commonly known as neomycin phosphotransferase II (NPTII), inactivates kanamycin, geneticin (G418) and neomycin sulphate by phosphorylation. In most plant transformation experiments, resistance to kanamycin is used to select transformed cells. However, it has been found that tissues of certain species, e.g. rice callus (Dekeyser *et al.*, 1989) display an endogenous tolerance to kanamycin which allows non-transformed cells to survive selection. To overcome this problem, cells transformed with nptII can be selected on media containing the kanamycin analogue G418, which is more toxic to the cells of some species than kanamycin. Alternatively, genes conferring resistance to other selection agents can instead be used in plant transformation experiments. Such genes include chimeric constructs conferring resistance to hygromycin (Waldron *et al.*, 1985), methotrexate (Dekeyser *et al.*, 1989), chloramphenicol (Umbeck *et al.*, 1989), bleomycin (Hille *et al.*, 1986) and some herbicides (see below). With the exception of bleomycin, which interferes with DNA synthesis, all of the antibiotics mentioned target protein synthesis at the ribosomal level (Maniatis *et al.*, 1982; Colbère-Garapin, 1981; Cabanas *et al.*, 1978).

In this study, responses of onion tissues to the antibiotics, kanamycin, G418 and hygromycin B, and to the herbicide chlorsulfuron were surveyed. Like kanamycin and G418, hygromycin is an aminoglycoside which is inactivated by a phosphorylation reaction mediated by the product of the hygromycin B phosphotransferase (hph) gene from *E. coli* (Waldron *et al.*, 1985). Chlorsulfuron, a sulphonylurea herbicide, is the active ingredient in the Du Pont product 'Glean'. It is a selective pre- and post-emergence herbicide, and like other sulphonylureas, inhibits the branched-chain amino acid biosynthetic enzyme acetolactate synthase (ALS) (Haughn *et al.*, 1988). Resistance to chlorsulfuron was first identified in a mutant cell line of *Nicotiana tabacum* (Chaleff and Ray, 1984), and subsequently in *Arabidopsis* plants (Haughn and Somerville, 1986). The mutant als gene (*csr1*), which differed from that of the wild type by a single base pair substitution, conferred a high level of resistance to chlorsulfuron. Resistance was inherited as a single dominant (or semi-dominant) mutation, making the gene suitable for use as a selectable marker. To determine

which selective agents would be toxic to onion tissues and at what concentrations, several types of onion tissues were grown *in vitro* on a range of kanamycin, G418, hygromycin and chlorsulfuron concentrations.

4.3. Materials and methods

All media containing antibiotics or chlorsulfuron were solidified with 0.7% agar (Davis). When autoclaved media had cooled to about 37°C, filter-sterilized PGRs, antibiotics and chlorsulfuron were added before pouring into tissue culture dishes. Unless otherwise stated, all cultures were subcultured after twelve weeks.

4.3.1 Germination and early seedling development on selective agents

'Pukekohe Longkeeper' ('PLK') and 'Early Longkeeper' ('ELK') seeds were surface sterilized in a 30% v/v solution of commercial bleach (5 % w/w sodium hypochlorite) and were plated on BDS basal medium (Dunstan and Short, 1977) without sucrose or PGRs. Kanamycin concentrations ranged from 0-500 mgl^{-1}, G418 from 0-150 mgl^{-1}, hygromycin from 0-100 mgl^{-1} and chlorsulfuron from 0-150 nM. Seeds were germinated in the light (cool, white fluorescent tubes, 30 $\mu E\ m^{-2}sec^{-1}$, 16 h daylength) or in the dark, at 20°C.

4.3.2 Callus formation and callus production

4.3.2.a *Kanamycin*

Surface-sterilized 'PLK' seeds were plated on callusing medium (BDS, 3% sucrose, 1.5 mgl^{-1} picloram) with added kanamycin (0-500 mgl^{-1}) and placed either in the light (cool, white fluorescent tubes, 30 $\mu E\ m^{-2}sec^{-1}$, 16 h daylength) or in the dark, at 20°C to germinate. After germination, seedlings were transferred to light or dark at 25°C. Callus produced from seedling stems was weighed 15 weeks later. Established 'PLK' callus was plated on callusing medium supplemented with concentrat-

ions of kanamycin ranging from 0-1000 mgl^{-1}.

4.3.2.b *Geneticin (G418)*

Excised seedling stems were used in G418 dose response experiments, as they were the explant type being used in *Agrobacterium* co-cultivation experiments at the time. Stems were excised from 10 day-old 'PLK', 'ELK' and 'SWG' seedlings, and plated on callusing medium with added G418 (0-150 mgl^{-1}). Explants were placed either in the light (cool, white fluorescent tubes, 30 $\mu E\ m^{-2}sec^{-1}$, 16 h daylength) or in the dark, at 25°C. Resulting callus was weighed 6 weeks later.

4.3.2.c *Hygromycin*

Surface-sterilized 'PLK' seeds were placed on callusing medium supplemented with hygromycin (0-100 mgl^{-1}). They were treated in the same way as seeds on kanamycin (section 4.3.2.a), except that callus produced was weighed after 11 weeks. As seeds usually took 2-3 weeks to germinate, this 11-week period of culture was approximately equivalent to the seven week culture period of excised seedling stems on callusing medium with G418. Established 'ELK' callus was also plated on callusing medium supplemented with the same concentrations of hygromycin used for callusing seedling stems.

4.3.2.d *Chlorsulfuron*

Excised 'PLK' seedling stems were placed on callus-induction medium supplemented with chlorsulfuron (0-150 nM). The method used was the same as that used for seedling stem callusing dose reponses on G418-supplemented media (section 4.3.2.b).

4.3.3 Shoot Culture

Effects of kanamycin, G418, hygromycin and chlorsulfuron on the growth and

multiplication of 'PLK', 'ELK' and 'SWG' shoot cultures were seen after growth of these cultures on shoot proliferation medium (section 3.3.4) supplemented with various concentrations of each selection agent. Concentrations used are listed in Appendices II, IV, V and VII. Shoot cultures on G418 and chlorsulfuron were assessed after 6 weeks, those on kanamycin after 8 weeks and those on hygromycin after 6 and 12 weeks.

4.3.4 Regeneration on hygromycin

'ELK' callus was plated on BDS medium containing 4 mgl^{-1} of thidiazuron (section 3.3.2.b) and concentrations of hygromycin ranging from 0-100 mgl^{-1}.

4.3.5 Statistical analysis

Where sample size was considered to be of sufficient size for statistical analyses, data were subjected to analysis of variance (ANOVA).

4.4. Results

4.4.1 Kanamycin

4.4.1.a *Germination and early seedling development*

On all concentrations of kanamycin surveyed, germination of 'PLK' seeds ranged from 55-100 percent (Appendix II). Germination itself was not noticeably inhibited by any concentrations of kanamycin in the light or in the dark, but seedlings cultured on 150-500 mgl^{-1} kanamycin were often bleached, and did not develop beyond the single cotyledon stage.

4.4.1.b *Seedling stem callus production*

Very little callus was produced by excised seedling stems on concentrations of kanamycin greater than 100 mgl^{-1}. Seedling stems were also bleached at these concentrations. On concentrations of 20 mgl^{-1} or greater, callus from light-grown stems weighed significantly less than that grown on control medium without kanamycin (Appendix II). In the dark, significantly less callus growth was seen on concentrations in excess of 100 mgl^{-1} (Appendix II). On 20-100 mgl^{-1} kanamycin in light and dark conditions, chlorosis and browning of seedling stems and of callus produced from them, first became apparent within nine weeks. Stems cultured in the dark on 1 mgl^{-1} kanamycin produced 47% more callus than dark-grown controls (Fig 4.1). Similarly, stems cultured in the light on 10 mgl^{-1} kanamycin produced 57% more callus than light-grown controls (Fig 4.1).

4.4.1.c *Growth of established callus*

Established 'PLK' callus was weighed after 24 weeks, when calli on concentrations of kanamycin ranging from 100-1000 mgl^{-1} were completely bleached and/or brown. Growth of callus was significantly inhibited on kanamycin concentrations of 50 mgl^{-1} or more (Appendix II). Although little callus growth occurred on concentrations of kanamycin more than 50 mgl^{-1}, 'PLK' callus only bleached and senesced within 12 weeks when cultured on media containing 1000 mgl^{-1} kanamycin.

4.4.1.d *Shoot cultures*

Shoot cultures required at least 12 weeks of culture on kanamycin before chlorosis and growth inhibition became apparent. Once tissue bleaching was initiated, leaves of shoot cultures senesced quickly (Fig 4.2; Appendix II).

4.4.2 G418

4.4.2.a *Germination and early seedling development*

The percentage of 'PLK' and 'ELK' seeds which germinated did not appear to be markedly decreased as G418 concentrations increased (Fig 4.3; Appendix III). Not all of the seedlings bleached and died within the 10 week period of the experiment.

4.4.2.b *Seedling stem callus production*

After culture on media supplemented with more than 25 mgl^{-1} G418, seedling stems of all three cultivars produced negligible amounts of callus in the light and in the dark (Fig 4.4; Appendix IV).

4.4.2.c *Shoot cultures*

The number of green leaves produced on media containing 10 mgl^{-1} or more of G418 was significantly less than that produced in control treatments. In addition, leaves produced on media supplemented with more than 10 mgl^{-1} were bleached by the end of the 11 week culture period (Fig 4.5; Appendix III). Vitrification was frequently seen in shoots grown on concentrations of G418 ranging from 5-30 mgl^{-1}. *In vitro* cultures used to start these shoot cultures had not previously been vitrified, and there was no evidence of vitrification in non-G418 controls. Roots produced by shoot cultures grown on lower concentrations of G418 (5-10 mgl^{-1}) were often noticeably greener than those of control plantlets. No roots were produced by shoots on media containing more than 30 mgl^{-1} G418.

4.4.3 Hygromycin

4.4.3.a *Germination and early seedling development*

The percentages of germinating 'ELK' seeds were high on medium containing 0-50 mgl^{-1} hygromycin. On medium supplemented with 100 mgl^{-1} hygromycin, some inhibition of germination was apparent (Appendix V). Germination of 'PLK' seeds appeared to be slightly inhibited on media with 10 mgl^{-1} or more of hygromycin (Appendix V). Seedlings on 20-100 mgl^{-1} of hygromycin were bleached and no

development occurred beyond the cotyledon stage (Plate 4.1). The average length of the hypocotyl, or of the first true leaf if produced, was significantly less than that of hygromycin-free controls when 'PLK' seeds were germinated in the presence of 20, 50 and 100 mgl^{-1} hygromycin (Appendix V).

4.4.3.b *Seedling stem callus production*

Seedlings usually grew to about 50mm in length before producing callus. Chlorosis of seedling tissue was apparent within approximately four weeks of germination (Plate 4.2). Average weights of seedling stem callus produced on 5-100 mgl^{-1} hygromycin was significantly less than that of non-hygromycin controls (Appendix V). No callus formed on 'PLK' or 'ELK' seedling stems when they were cultured on concentrations of hygromycin in excess of 20 mgl^{-1} (Plate 4.2; Appendix V).

4.4.3.c *Established callus*

Chlorosis of callus cultured on 20-100 mgl^{-1} hygromycin was apparent within six weeks, and all calli on 50 and 100 mgl^{-1} had senesced within 11 weeks (Plate 4.3; Appendix V).

4.4.3.d *Shoot cultures*

Whereas shoot cultures grown on media containing 0 and 5 mgl^{-1} hygromycin produced green leaves, those on all other treatments were bleached within 6 weeks. This experiment was continued for a further 6 weeks to check that no new shoots were produced. The number of bleached or brown leaves visible on shoots which were grown on 5, 10, 20 and 50 mgl^{-1} hygromycin, was significantly higher than on the hygromycin-free controls. Very few leaves were produced on 100 mgl^{-1} hygromycin (Fig 4.6; Appendix V). Vitrification was occasionally apparent in shoot cultures on hygromycin.

4.4.3.e *Regeneration*

No shoots were regenerated from calli growing on thidiazuron-supplemented medium, including those growing in the absence of hygromycin. Calli growing on 0, 5, 10 and 20 mgl^{-1} hygromycin produced roots. Some of the roots produced from callus growing on 5 mgl^{-1} hygromycin were green (Plate 4.4). No callus grew on media containing 50 or 100 mgl^{-1} hygromycin.

4.4.4 Chlorsulfuron

4.4.4.a *Germination and early seedling development*

Although germination of 'PLK' and 'ELK' seeds did not appear to be inhibited by high concentrations of chlorsulfuron, subsequent growth of seedlings was (Plate 4.5; Appendix VI). Seedlings on concentrations of chlorsulfuron above and including 50 nM were mostly bleached within 11 weeks of the seeds being put onto germination media. On concentrations above and including 50 nM, the percentage of bleached seedlings ranged from 73 to 100% ('ELK') and from 43 to 75% ('PLK'). The percentage of bleached seedlings increased with increasing chlorsulfuron concentrations (Fig 4.7).

4.4.4.b *Seedling stem callus production*

The weight of callus produced by excised seedling stems cultured in the light was significantly less than non-chlorsulfuron controls when explants were grown on 25-150 nM chlorsulfuron (Fig 4.8; Appendix VI). A similar result was recorded in the dark (Appendix VI). Negligible callus growth was recorded for seedling stems on 25-150 nM chlorsulfuron.

4.4.4.c *Shoot cultures*

The number of leaves produced by 'PLK', 'ELK' and 'SWG' shoot cultures within six weeks, was significantly less than non-chlorsulfuron controls for cultures grown on all concentrations of chlorsulfuron (Fig 4.9; Appendix VII). Shoot cultures of

all three cultivars produced an average of less than one leaf on concentrations of chlorsulfuron greater than and including 25 nM. With the exception of 'ELK' on 25 and 100 nM chlorsulfuron, the number of roots produced by shoot cultures grown on all of the cultivar-chlorsulfuron combinations was significantly less than that of controls (Appendix VII). Roots produced by plantlets growing on 25 and 50 nM chlorsulfuron were greener than those of control plantlets.

4.5. Discussion

4.5.1 General

Dose response experiments were carried out to determine the minimum concentrations of selection agents which could be used for selection of transformed onion cells (Table 4.1). This would avoid the use of excess antibiotic or herbicide in selection media, which might otherwise result in the death of transformed cells. Death of transformed cells might be caused by excess antibiotic in one of two possible ways: firstly, leachates e.g. phenolics and other compounds, produced by surrounding untransformed cells when they die on selection media, can cause the death of viable transformed cells; secondly, transformed cells in the co-cultivated plant tissue are only likely to tolerate as much toxic compound as can be detoxified by the enzyme for which the selectable marker gene codes. Saturation of this enzyme with substrate (i.e. the selective agent) may result in cell death.

4.5.2 Effects of kanamycin, G418 and hygromycin

4.5.2.a *Germination and early seedling development*

Germination/seedling growth dose response experiments generally showed that the

Table 4.1 Concentrations of selection agents suitable for use in selection of transformed 'Pukekohe Longkeeper' tissues.

Tissue Type	Selection Agent			
	Kanamycin	Geneticin	Hygromycin	Chlorsulfuron
Seedling stem (shoot production)	100 mgl^{-1} (n.a.) (light)	25 mgl^{-1} (n.a.)	5 mgl^{-1} (n.a.)	25 nM (n.a.)
Seedling stem (callus production)	100 mgl^{-1}*** (light)	25 mgl^{-1} (n.a.)	5 mgl^{-1}***	25 nM (light)
Shoot cultures	100 mgl^{-1} (n.a.) (light)	30 mgl^{-1}**	10 mgl^{-1}***	25 nM ***
Mature callus	600 mgl^{-1}***	30 mgl^{-1} (n.a.)	20 mgl^{-1} (n.a.)	not tested
Regenerating callus	600 mgl^{-1}***	not tested	20 mgl^{-1} (n.a.)	not tested

N.B. Statistical comparisons are made with controls following analyses of variance combined with Fisher's least-significant-difference test (*, **, *** = significance at 0.05, 0.01 and 0.001 levels respectively; n.a. = data not analysed statistically).

actual germination event was not directly inhibited by kanamycin, G418 or hygromycin. As kanamycin, G418 and hygromycin interfere with ribosomal components of the cell, thereby inhibiting protein synthesis (Maniatis *et al.*, 1982; Colbère-Garapin, 1981; Cabanas *et al.*, 1978), their presence in the medium probably does not greatly affect pre-formed meristems already in place in the seed. In most work done on screening progeny of kanamycin-resistant plants transformed with the nptII gene, workers have found germination of seeds to be unaffected by kanamycin (Horsch *et al.*, 1984; Deroles and Gardner, 1988). Conversely, seedling growth is usually affected by kanamycin, and growth of seedlings on kanamycin-containing media is used to screen for inheritance of nptII (Horsch *et al.*, 1984).

After germination, onion seedlings grown on higher concentrations (150-500 mgl^{-1}) of kanamycin, were bleached and growth-impaired. By comparison, G418, although noticeably more toxic to onion shoot cultures than kanamycin, did not appear to affect viability of seedlings as much as kanamycin. It appears that these two analogues affect different developmental stages of onions in different ways, with kanamycin being more toxic to seedlings and G418 to more toxic to mature plants. As higher concentrations of hygromycin (50 and 100 mgl^{-1}) did not facilitate any growth of seedlings (Plate 4.1), this antibiotic could be reliably used to screen progeny of onion plants transformed with hpt.

4.5.2.b *Callus growth*

Of all the onion tissues surveyed for responses to kanamycin, callus was the least sensitive. At concentrations greater than and including 50 mgl^{-1}, hygromycin was more toxic to seedling stem cells than kanamycin or G418, as no callus was formed. By comparison, seedling stems produced some callus when grown on 50 mgl^{-1} kanamycin, and a smaller amount on 50 mgl^{-1} G418. The complete lack of seedling stem callus formation on hygromycin, and the marked growth inhibition and bleaching of established callus, suggest that hygromycin is more effective than G418 to use in selection of transformed onion callus. Regeneration of plantlets from transformed callus should also be carried out on media containing hygromycin. Unlike

kanamycin, which has been shown to inhibit regeneration of kanamycin-resistant calli when added to regeneration media of some species e.g. asparagus (Conner *et al.*, 1988), potato (Conner *et al.*, 1991b) and pea (Puonti-Kaerlas *et al.*, 1990), hygromycin has not been shown to inhibit regeneration of hygromycin-resistant calli when included in regeneration media of maize (Walters *et al.*, 1992), tobacco (Zyprian and Kado, 1990) and pea (Puonti-Kaerlas *et al.*, 1990).

In these dose response experiments, growth of young and established onion callus has been shown to be only partially inhibited even on high concentrations of kanamycin. This has also been noted with callus of other species. Dekeyser *et al.* (1990) showed that although growth of rice callus is sensitive to low concentrations of methotrexate, phosphinothricin and bleomycin, and to moderate concentrations of G418 and hygromycin, it is only partially inhibited by relatively high concentrations of kanamycin. By comparison, callus of wheat, which like rice is also a member of the Poaceae (Gramineae) family, is sensitive to moderate amounts of kanamycin. Mooney *et al.* (1991) selected for transformed kanamycin-resistant wheat calli on 100 mgl^{-1} kanamycin, this dose being toxic to untransformed cells. It is clear from these and other results, that tolerance/sensitivity to selective agents must be defined for each particular species and cultivar being used, prior to transformation experiments being performed.

The hormone-like enhancement of onion callus growth on low concentrations of kanamycin, as was seen in these experiments, has also been observed by other workers. Owens (1979) found that low concentrations of kanamycin (2.9-11.65 mgl^{-1}) enhanced shoot differentiation from carrot callus cultures. The presence of kanamycin was necessary for shoot morphogenesis from callus of one of the cultivars. The concentrations of kanamycin which enhanced morphogenesis from carrot callus are similar to those which enhanced onion callus growth, i.e., 1-10 mgl^{-1}. It is possible that the promotion of onion callus growth at low concentrations of kanamycin was not a response of the cells to kanamycin, but rather a response of cells to suppressed growth of bacterial contaminants. If any contaminating bacteria were suppressed, improved callus growth would probably result.

4.5.2.c *Regeneration*

Regeneration of shoots or whole plantlets from 'ELK' callus on hygromycin was not successful even on hygromycin-free controls. It is thought that regeneration did not occur on control tissue because the callus was more than six months old, after which time 'ELK' and 'PLK' callus had generally lost its ability to regenerate shoots or plantlets (see section 3.4.2).

4.5.2.d *Shoot cultures*

Kanamycin was shown to be ineffective as a selection agent in onion shoot culture dose response experiments. The waiting period of at least 12 weeks before it was clear if plants had bleached and senesced, was considered too long. Consequently, G418 was chosen as an alternative antibiotic for use in the selection of tissue thought to be transformed with the nptII gene. The modes of action of these two analogues should be noted here: although kanamycin and G418 both interfere with ribosomal componentry of cells, kanamycin binds to 70S ribosomes (Cabanas *et al.*, 1978; Colbère-Garapin *et al.*, 1981; Maniatis *et al.*, 1982), which are found in bacteria and in the mitochondria and chloroplasts of plant cells, and G418 interferes with the function of 80S ribosomes, i.e. eukaryote cell cytoplasmic ribosomes (Eustice and Wilhelm, 1984), blocking protein synthesis in the cell. This may account for the difference in reponses of onion leaf tissue to the two antibiotics.

The reason for the vitrification response of leaves to G418 is unclear. Many theories have been put forward to explain this glassy, watery condition of shoots in tissue culture. Some of the more common ones include deficient lignin synthesis, lack of enzyme activity (Phan and Hegedus, 1986), the presence of ethylene, the physical state of the culture medium and the presence of cytokinins (Leshem *et al.*, 1988; Phan, 1991). It is possible that stress on shoots caused by the presence of G418, combined with stress caused by one or several of the above factors, could have been responsible for the widespread vitrification of leaf tissue. As vitrification had been occasionally seen in some onion shoot cultures not on G418, BA and sucrose

concentrations in the onion shoot proliferation medium had already been reduced. Cultures had also been regularly subcultured (approximately every 6 weeks) to avoid the build up of ethylene in culture vessels.

Growth of onion shoot cultures on hygromycin-supplemented media established that 10 mgl^{-1} was a suitable concentration on which to select for hpt-positive shoots. This concentration would allow maximum growth from inoculated basal plates at first, allowing transformed shoots to get established, and would then cause any untransformed shoots subsequently produced in culture to senesce.

4.5.3 Chlorsulfuron

From the data presented here, it would appear that chlorsulfuron is a suitable selective agent for use in onion transformation experiments. If selection for chlorsulfuron resistance were to be carried out during callusing, a concentration of 25 nM would be sufficient. This low concentration required for selection demonstrates the toxicity of chlorsulfuron to onion cells. The pKIWI110 binary vector, which contains the mutant als gene in its T-DNA, was initially used in onion transformation experiments described in Chapter 5. However, since pKIWI110 was shown by Gardner and Janssen (pers. comm.) to be suboptimal in its delivery of T-DNA to plants usually readily transformable by *Agrobacterium*, it has been abandoned as a binary vector for use in onion transformation (for more detail see section 5.5).

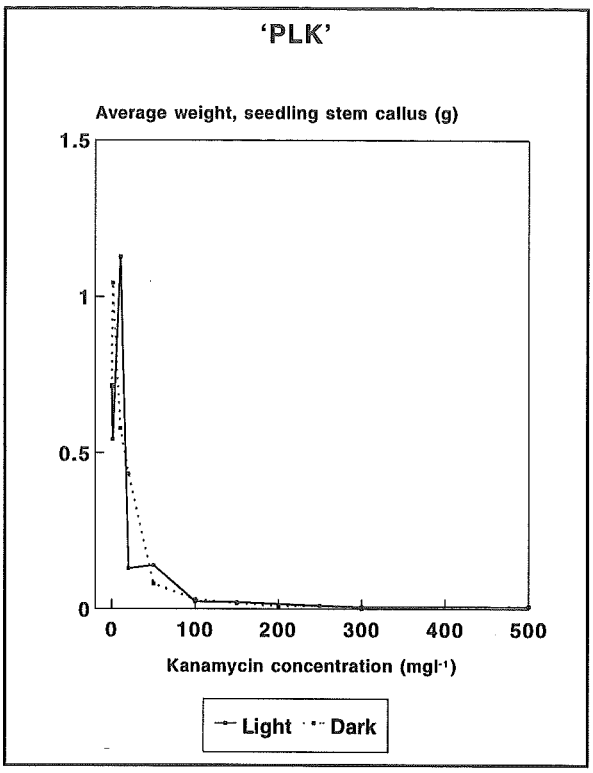
4.5.4 Concluding remarks

The dose response experiments carried out showed that kanamycin was ineffective as a selective agent for use in onion transformation work. By comparison, the kanamycin analogue G418 was relatively effective, causing the death of most onion tissues at low concentrations. However, responses of tissues to G418 were slower than those to the antibiotic hygromycin. Most tissue types died within two months on 30 mgl^{-1} G418, but young and mature callus could survive on this concentration

for up to six months. Hygromycin was the most toxic antibiotic to 'PLK' onion tissue cultures. No callus grew from seedling stems cultured on 20 mgl^{-1} or more of hygromycin, and all leaves of *in vitro* cultures grown on hygromycin were bleached and dead within eight weeks. These responses suggest that hygromycin is even more preferrable as an antibiotic selectable marker for use in onion transformation experiments than G418. However, one disadvantage of using the hpt gene for selection is that although an assay to detect HPT activity in plant tissue is now available (Cabanes-Bastos *et al.*, 1989; Spangenberg *et al.*, 1991), this assay is time-consuming, and is not routinely used to screen for transformed plants. Because the hygromycin dose response work was carried out late in this project, transformation of onions with an hpt expression vector was not attempted (G418-resistant plantlets had already been selected by this time). The sensitivity of onion tissues to low concentrations (25-150 nM) of chlorsulfuron demonstrated that this herbicide would also be a suitable selective agent in transformation experiments.

Fig 4.1 The average weight of 'Pukekohe Longkeeper' seedling stem callus produced on medium containing kanamycin. Seedling stems were cultured in the light and the dark for 15 weeks.

Fig 4.2 The average number of green leaves produced by 'Pukekohe Longkeeper' shoot cultures on medium containing kanamycin. Shoots were cultured for 8 weeks.


'PLK'
Average weight, seedling stem callus (g)
1.5
1
0.5
0
0
100
200
300
400
500
Kanamycin concentration (mgl-1)
Light
Dark

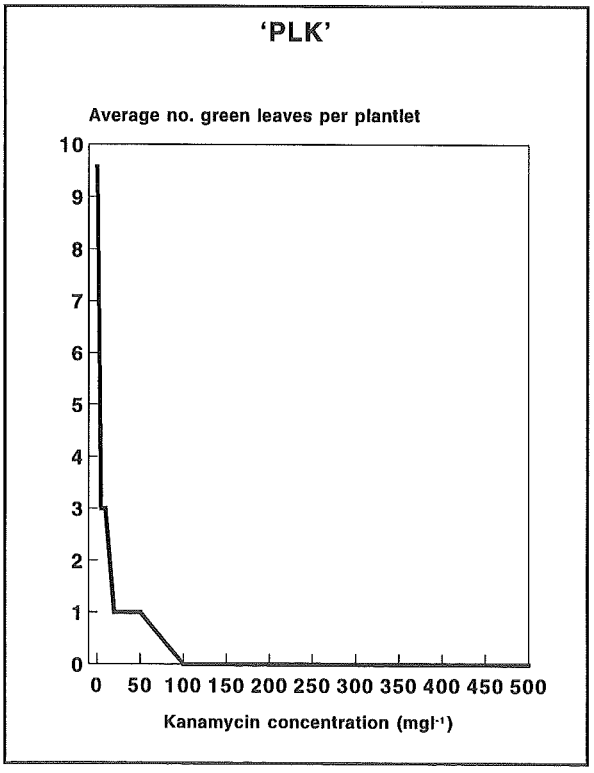
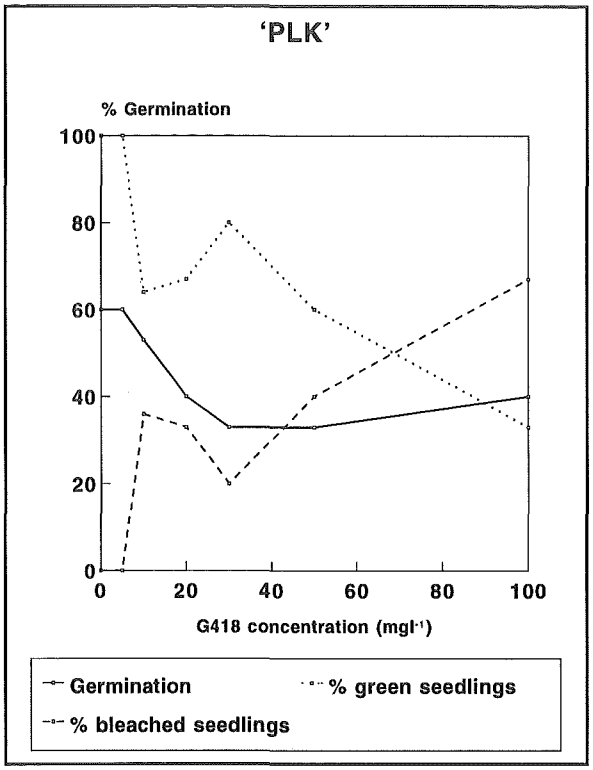
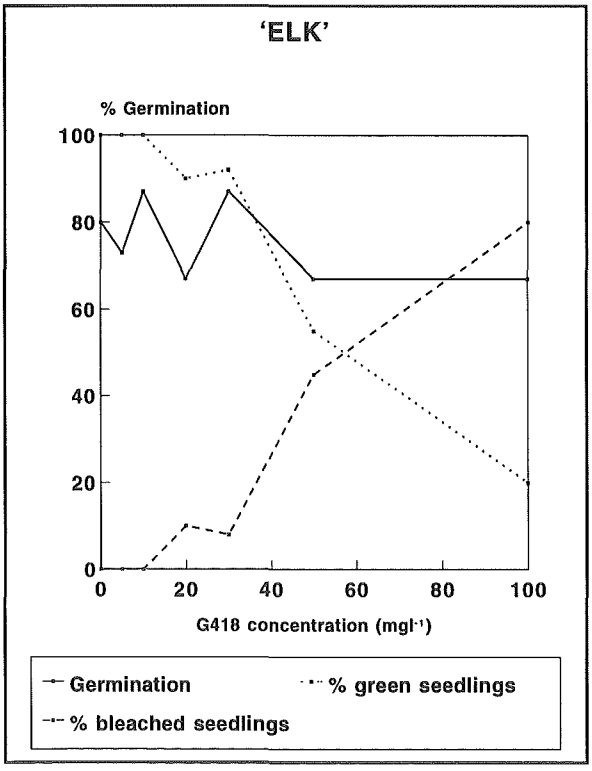

'PLK'
Average no. green leaves per plantlet
10
9
8
7
6
5
4
3
2
1
0
0 50 100 150 200 250 300 350 400 450 500
Kanamycin concentration (mgl-1)

Fig 4.3 Germination of seeds and seedling growth of (a) 'Pukekohe Longkeeper' and (b) 'Early Longkeeper' on medium containing G418. Data was collected 10 weeks after seeds were plated.


'PLK'
% Germination
100
80
60
40
20
0
0
20
40
60
80
100
G418 concentration (mgl-1)
Germination
% green seedlings
% bleached seedlings

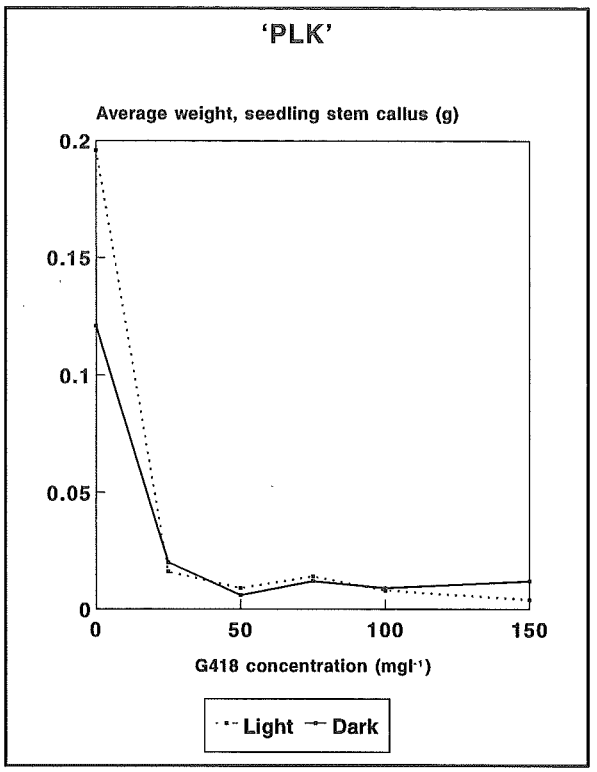
(a)


'ELK'
% Germination
100
80
60
40
20
0
0
20
40
60
80
100
G418 concentration (mgl-1)
Germination
% green seedlings
% bleached seedlings

(b)

Fig 4.4 The average weight of callus produced by light and dark-cultured 'Pukekohe Longkeeper' seedling stems on medium containing G418. Callus was cultured for 6 weeks.

Fig 4.5 The average number of green leaves and roots produced by 'Pukekohe Longkeeper' shoot cultures on medium containing G418. Shoots were cultured for 6 weeks.


'PLK'
Average weight, seedling stem callus (g)
0.2
0.15
0.1
0.05
0
0
50
100
150
G418 concentration (mgl⁻¹)
Light
Dark

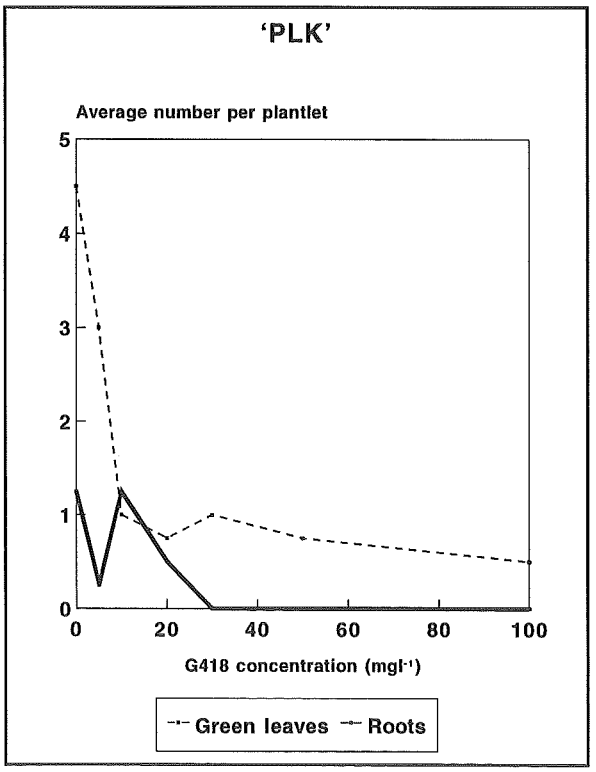

'PLK'
Average number per plantlet
5
4
3
2
1
0
0
20
40
60
80
100
G418 concentration (mgl⁻¹)
Green leaves
Roots

Fig 4.6 The average number of green and bleached leaves produced by 'Pukekohe Longkeeper' shoot cultures on medium containing hygromycin. Shoots were cultured for 12 weeks.

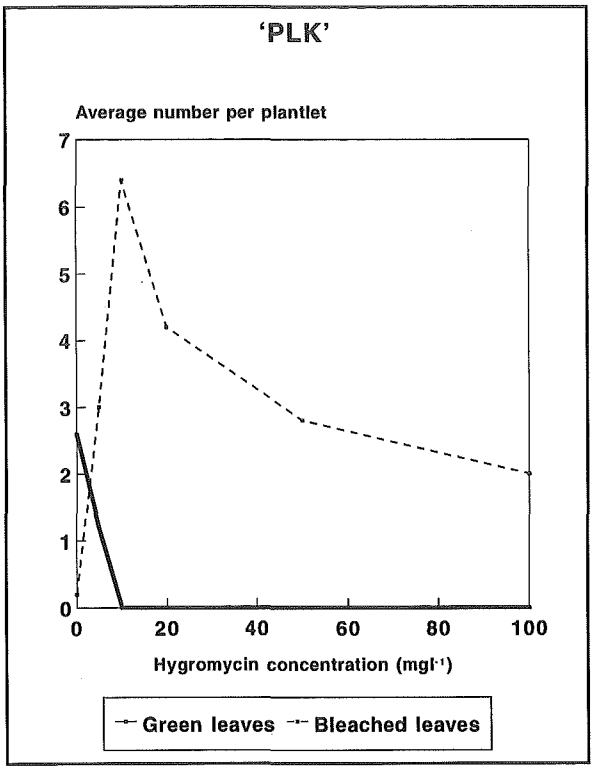
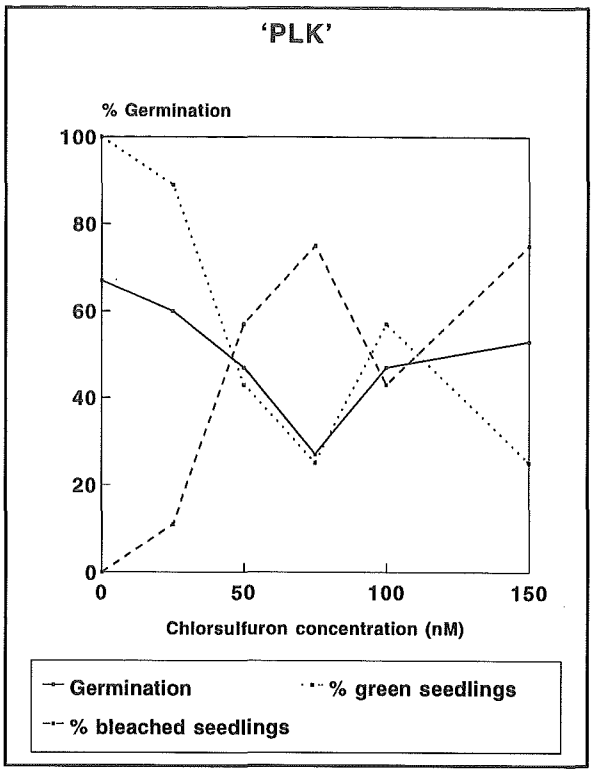
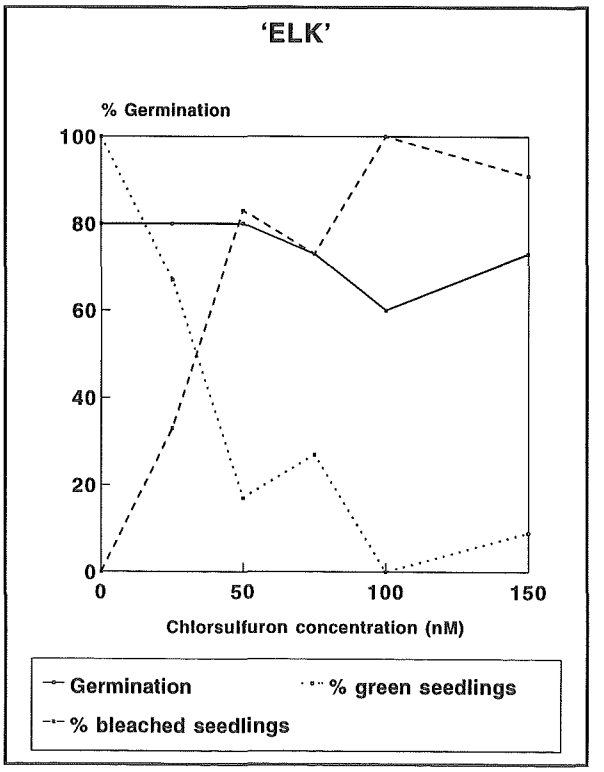

'PLK'
Average number per plantlet
7
6
5
4
3
2
1
0
0
20
40
60
80
100
Hygromycin concentration (mgl-1)
Green leaves
Bleached leaves

Fig 4.7 Germination of seeds and seedling growth of (a) 'Pukekohe Longkeeper' and (b) 'Early Longkeeper' seedlings on medium containing chlorsulfuron.


'PLK'
% Germination
100
80
60
40
20
0
0
50
100
150
Chlorsulfuron concentration (nM)
Germination
% green seedlings
% bleached seedlings

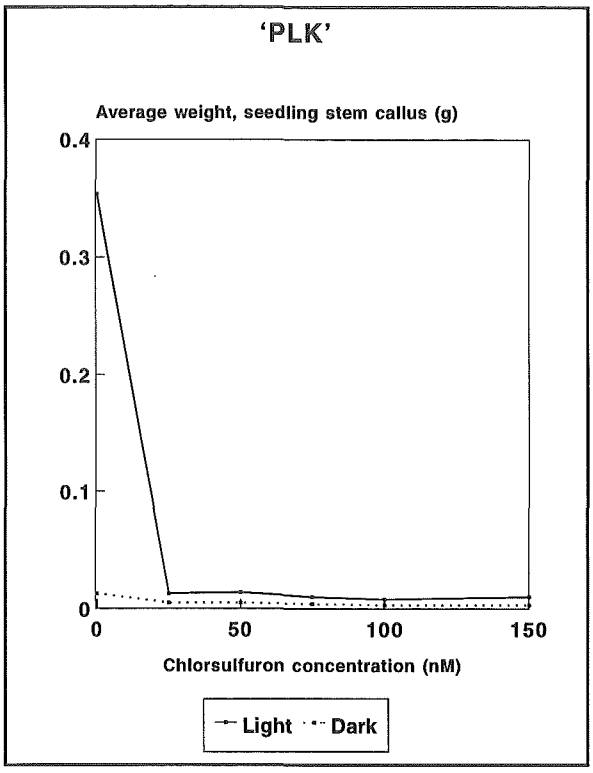
(a)


'ELK'
% Germination
100
80
60
40
20
0
0
50
100
150
Chlorsulfuron concentration (nM)
Germination
% green seedlings
% bleached seedlings

(b)

Fig 4.8 Germination and production of callus from light and dark-cultured 'Pukekohe Longkeeper' seedling stems on medium containing chlorsulfuron. Seedling stems were cultured for 6 weeks.

Fig 4.9 Leaf and root production by 'Pukekohe Longkeeper' shoot cultures on medium containing chlorsulfuron. Shoots were cultured for 6 weeks.


'PLK'
Average weight, seedling stem callus (g)
0.4
0.3
0.2
0.1
0
0
50
100
150
Chlorsulfuron concentration (nM)
Light
Dark

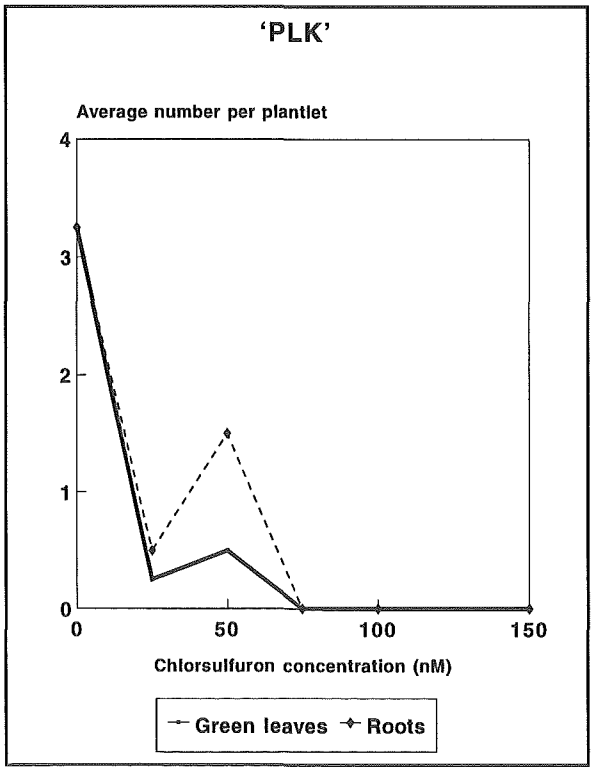
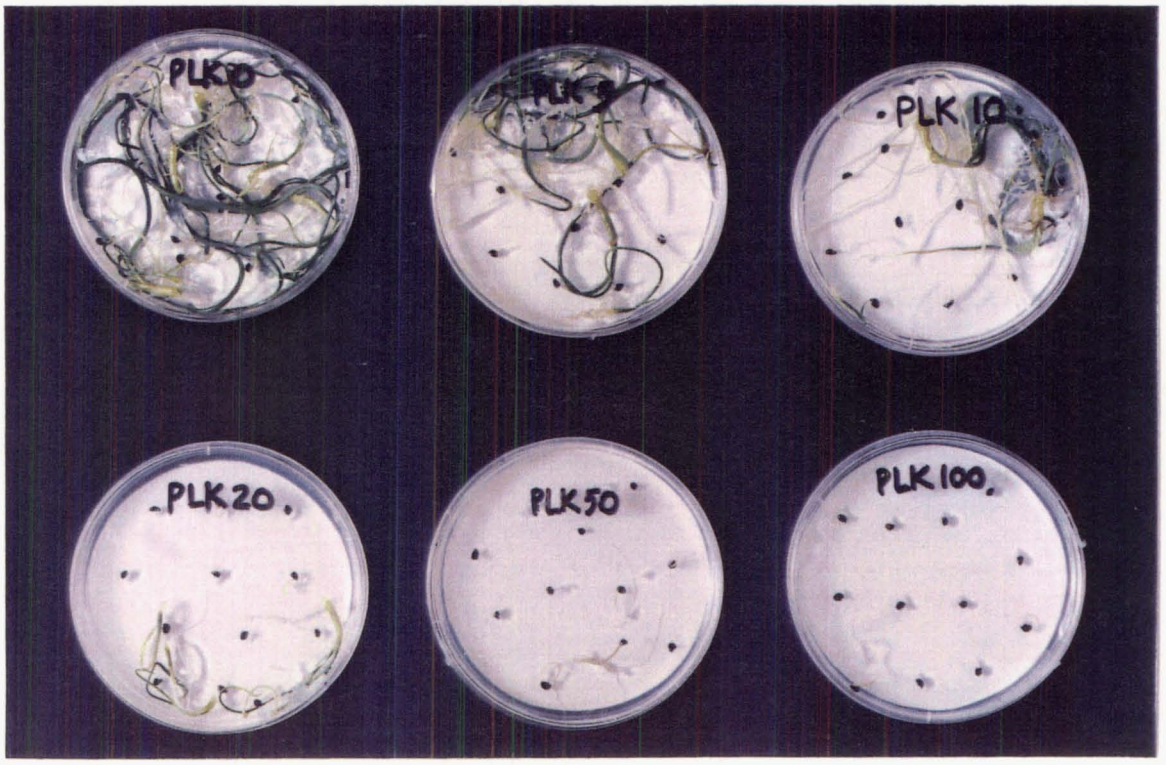

'PLK'
Average number per plantlet
4
3
2
1
0
0
50
100
150
Chlorsulfuron concentration (nM)
Green leaves
Roots

Plate 4.1 Germination of 'Pukekohe Longkeeper' (a) and 'Early Longkeeper' (b) seeds and seedling growth on medium containing (from left, top) 0, 5, 10, (bottom) 20, 50, and 100 mgl^{-1} hygromycin. Photographs were taken 11 weeks after seeds were plated.

a


PLK 0
PLK 5
PLK 10
PLK 20
PLK 50
PLK 100

b

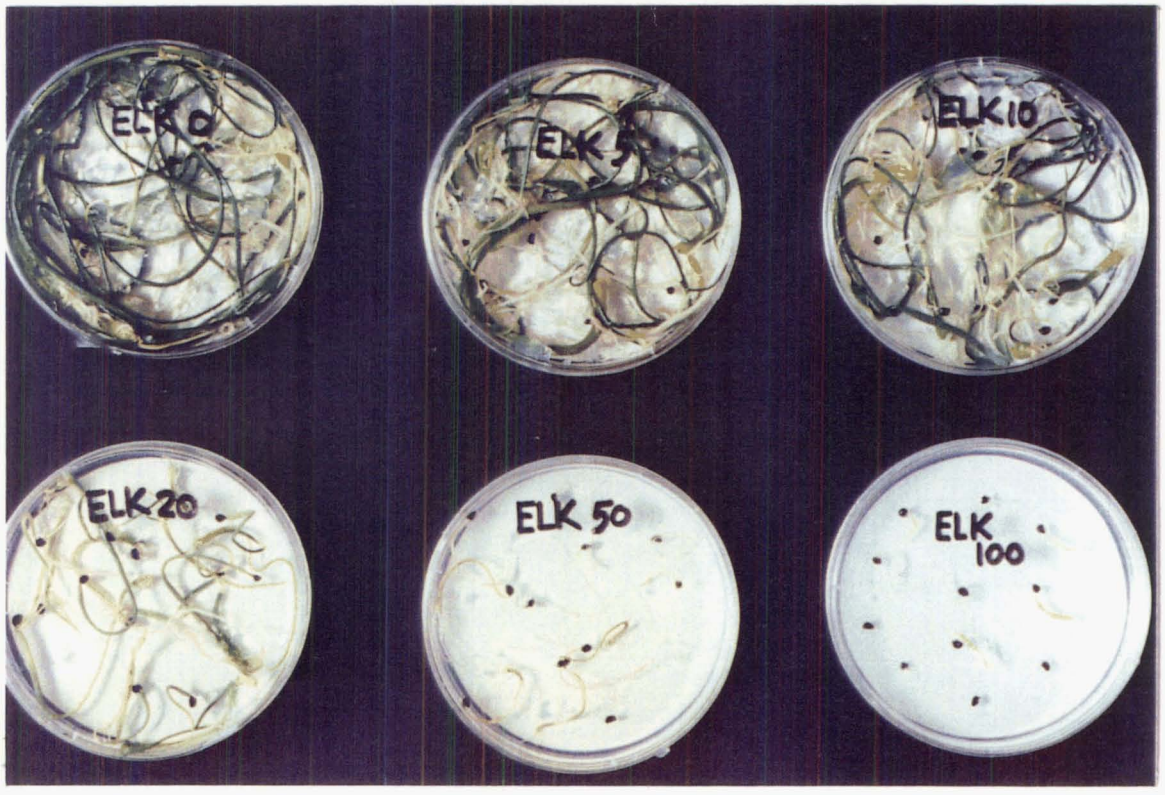
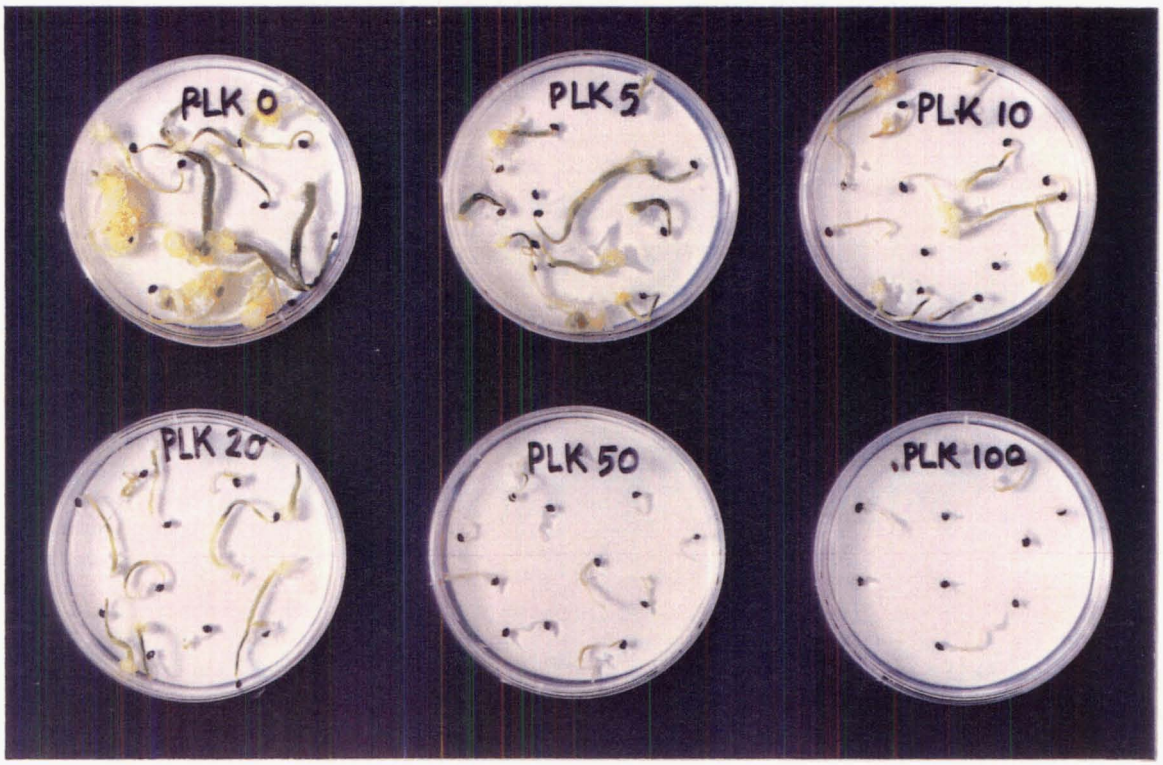

ELK 0
ELK 5
ELK 10
ELK 20
ELK 50
ELK 100

Plate 4.2 Germination of 'Pukekohe Longkeeper' seeds and callus production from seedling stems on medium containing (from left, top) 0, 5, 10, (bottom) 20, 50, and 100 mgl^{-1} hygromycin.

Plate 4.3 Growth of established 'Early Longkeeper' callus on medium containing (from left, top) 0, 5, 10, (bottom) 20, 50, and 100 mgl^{-1} hygromycin.

Both photographs were taken 11 weeks after seeds were plated.


PLK 0
PLK 5
PLK 10
PLK 20
PLK 50
PLK 100

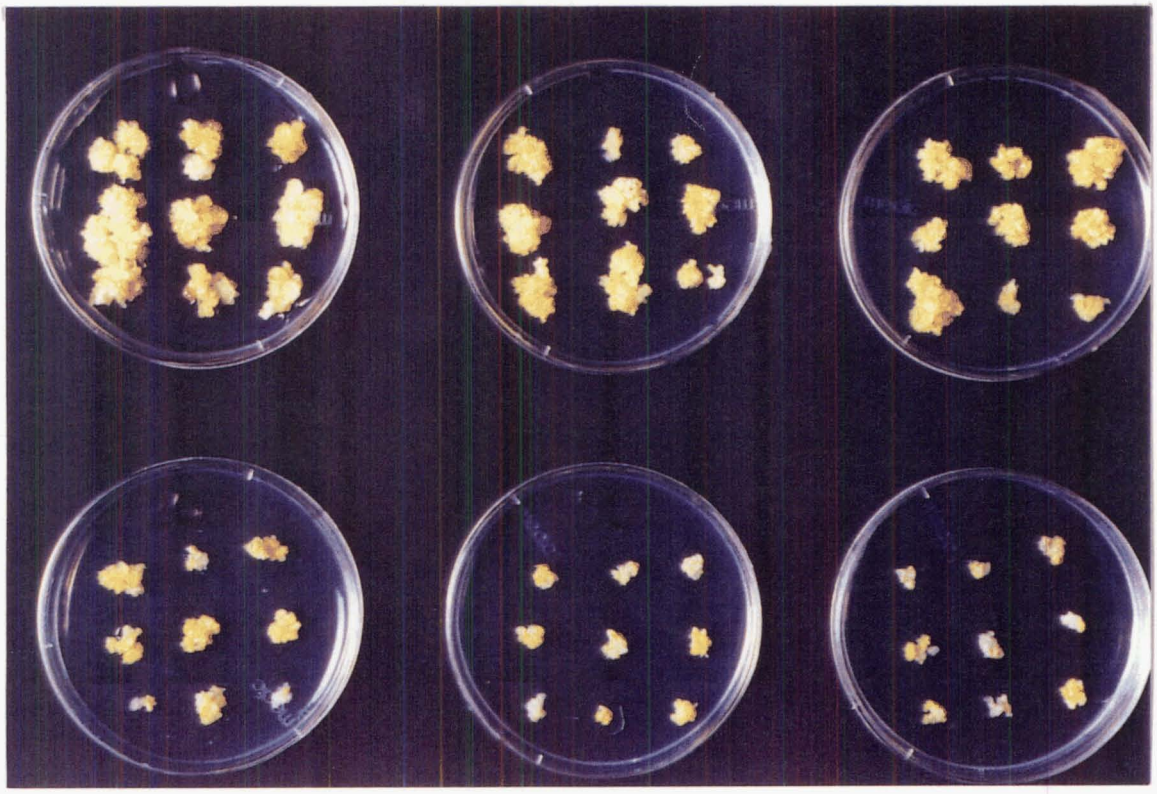
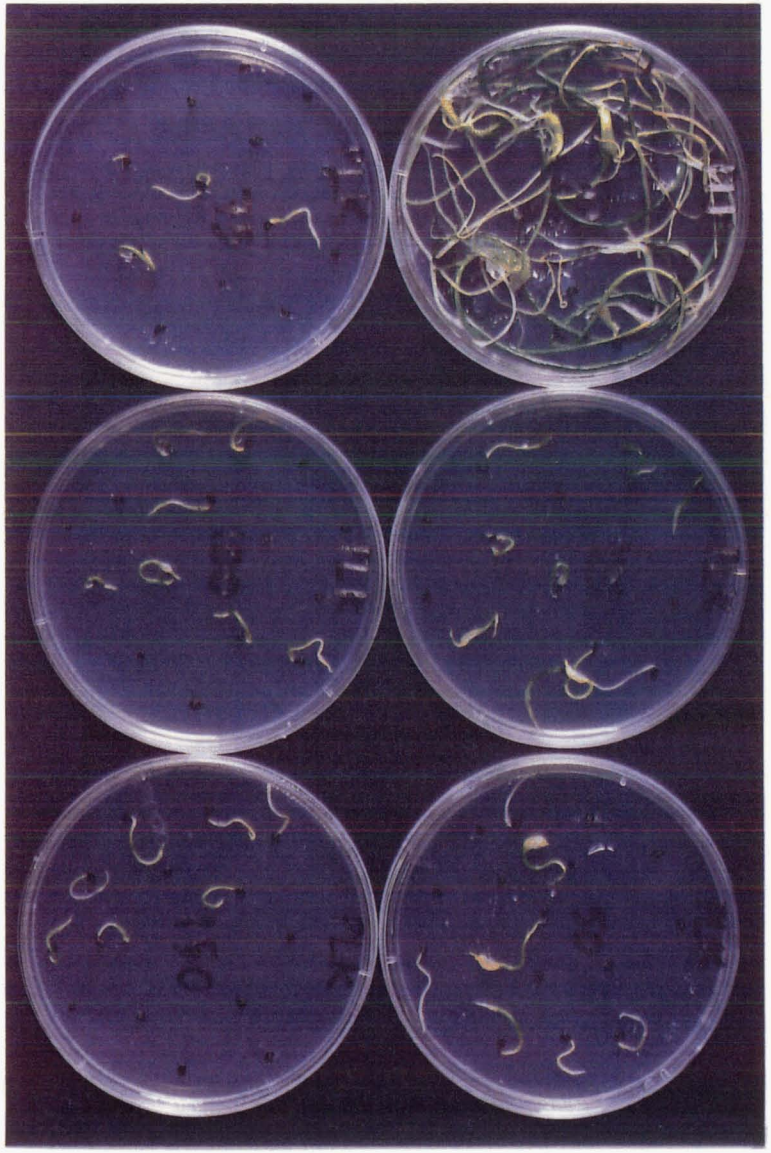
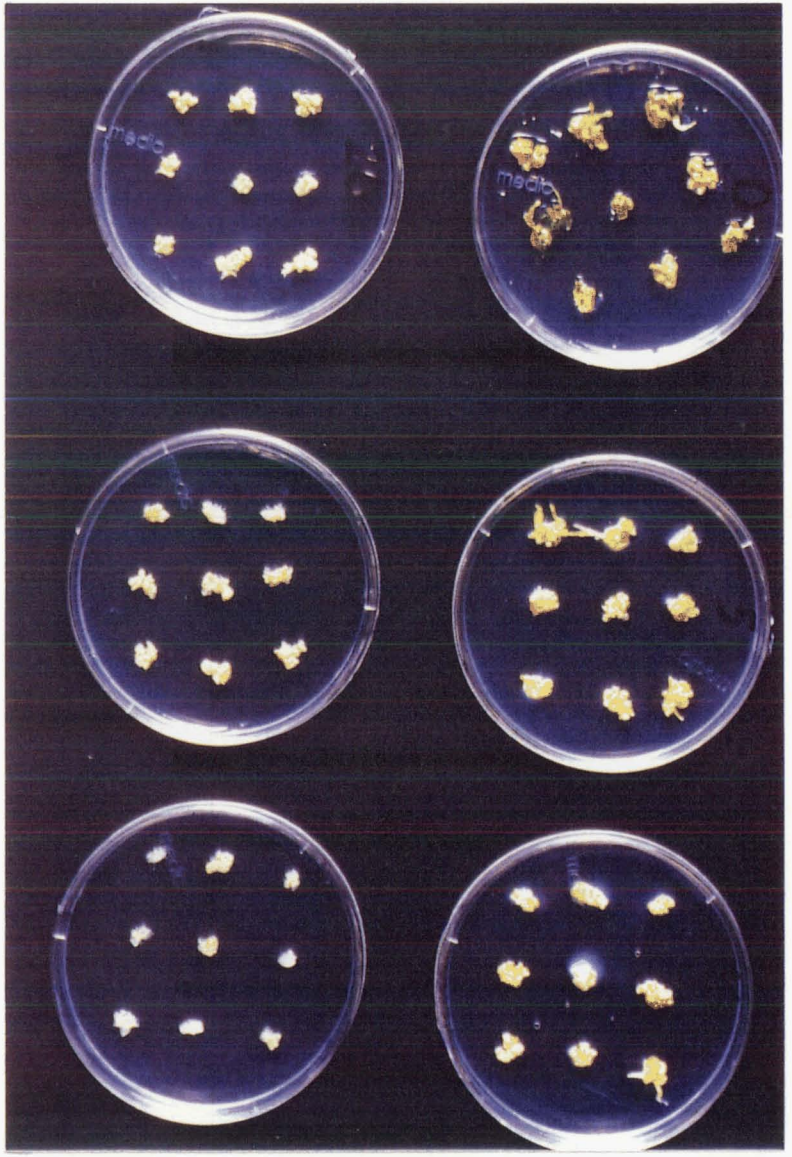


Plate 4.4 Growth of mature 'Early Longkeeper' callus on thidiazuron regeneration medium containing (from left) 0, 5, 10, (top) 20, 50, and 100 mgl^{-1} (bottom) hygromycin.

Plate 4.5 Germination of 'Pukekohe Longkeeper' seeds, and seedling growth on medium containing (from left) 0, 25, 50, (top) 75, 100 and 150 nM (bottom) chlorsulfuron.

Both photographs were taken 11 weeks after seeds were plated.




media
media

Chapter 5: Gene transfer to onion (*Allium cepa*) by *Agrobacterium tumefaciens*

5.1. Abstract

The ability of *Agrobacterium tumefaciens* to transfer genes to a monocotyledonous plant species has been demonstrated in onion (*Allium cepa* L.). A single, putatively transformed plantlet (RC1) was regenerated from onion seedling stem callus, following co-cultivation of seedling stems with *Agrobacterium* strain LBA4404 harbouring the binary vector pKIWI110. In addition, 41 axillary and/or adventitious shoots which grew directly from *in vitro*-injected basal plate meristematic regions, survived three cycles of selection on 30 mgl^{-1} of the kanamycin analogue G418 (geneticin) following co-cultivation with four strains of *A. tumefaciens*, each harbouring one of the two binary vectors, pKIWI110 or pGA643. This concentration of G418 is normally toxic to untransformed shoot cultures. The binary vectors pKIWI110 and pGA643 carry the neomycin phosphotransferase II gene (nptII) controlled by the nopaline synthase (*nos*) promoter. Both RC1 and the shoots growing from basal plate explants, produced roots when grown on culture media supplemented with 30 mgl^{-1} G418. DNA extracted from RC1 and from some of the G418-resistant shoots produced from injected basal plates, was shown by Southern hybridizations to contain the nptII gene, the gene product of which confers resistance to kanamycin and G418. β-glucuronidase (GUS) activity was detected in some of the plantlets derived from basal plate tissue which had been injected with the *A. tumefaciens* strains LBA4404 and C58 harbouring pKIWI110. Molecular and phenotypic evidence suggest that the putatively transformed plants produced from injected basal plate tissues are chimeric.

5.2. Introduction

The most common approach used to produce transgenic plants is that of co-cultivating sterile plant tissue with log phase cultures of genetically modified *Agrobacterium*. Plant protoplasts were used in early *Agrobacterium* co-cultivation experiments (Horsch *et al.*, 1984; De Block *et al.*, 1984; see section 1.5.2). The technique of leaf-disc co-cultivation was first used for *Agrobacterium*-mediated transformation of the solanaceous species petunia, tobacco and tomato by Horsch *et al.* (1985). Leaf discs, cut from surface-sterilized leaves were dipped into a suspension of *A. tumefaciens* harbouring a chimeric *nos*-nptII-*nos* gene in the T-DNA (section 1.5.2). After being blotted dry, leaf discs were incubated for two days on regeneration medium with feeder cells to stimulate growth. They were then transferred to the same medium without feeder cells, but with added carbenicillin to inhibit *Agrobacterium* growth, and kanamycin to select for transformed cells. Transformation of regenerated plants was confirmed by nopaline analysis, Southern blots and Mendelian inheritance patterns of the segregation of kanamycin resistance in seedling progeny. Since these experiments, co-cultivation has become a routine transformation technique used for plant species that are susceptible to *Agrobacterium* infection and can be readily regenerated from explant tissue. Some explant types used for this technique have included leaves, cotyledons, thin cell layers, petioles, peduncles, hypocotyls, stems, microspores and pro-embryos (Grant *et al.*, 1991).

Onions are among a small number of monocotyledonous plants which have been found capable of producing true tumours in response to *Agrobacterium* inoculation (Dommisse *et al.*, 1990; see Table 1.1, for detail on other monocotyledonous plants which have formed tumours in response to inoculations with *Agrobacterium*). The detection of opines in these tumours has provided additional evidence for expression of T-DNA genes, indicating that T-DNA has probably been integrated into the genome (Dommisse *et al.*, 1990; see Chapter 2). It should therefore be possible to regenerate transformed onion plants from excised, wounded tissue which has been

co-cultivated with *Agrobacterium*. The work reported in this chapter describes a number of approaches taken in an attempt to select and regenerate transformed onion cell cultures. Evidence for the presence and expression of nptII and gus in the onion genome is examined.

5.3. Materials and Methods

5.3.1 Bacterial strains and expression vectors

Explants for co-cultivation and injection were inoculated with *A.tumefaciens* strain LBA4404, C58, A4T or A281, each of which was carrying one of the binary vectors pKIWI110 or pGA643. The avirulent strain LBA4404 without a binary vector was used as the control strain in all co-cultivations performed. Simplified diagrams of the T-DNA regions of pKIWI110 and pGA643 can be seen in Fig 5.1 and Fig 5.2. A brief description of each of the vectors follows:

(1) pKIWI110: a complete description of this vector's construction is given elsewhere (Janssen and Gardner, 1989). Briefly, its T-DNA contains a chimeric gus gene (35S-gus-ocs) from pRAJ275 (Jefferson, 1987), constructed so that it is expressed in transformed plant tissues but not in bacterial cells. Closer to the T-DNA's right border is a chimeric kanamycin resistance gene (*nos*-nptII-*nos*). Also on the T-DNA is a mutant acetolactate synthase gene (als), the gene product of which confers resistance to sulfonylurea herbicides, e.g., chlorsulfuron.

(2) pGA643: this binary vector is based on an RK2 derivative, pTJS75 (An *et al.*, 1988). The nptII coding region from Tn5 is inserted between the nopaline synthase (*nos*) promoter and terminator regions. Although nptII is the only selectable marker on pGA643, this binary vector contains the DNA fragment carrying the transcript 7 and 5 terminators of the octopine-type Ti plasmid pTiA6 and the 419 bp DNA fragment carrying the 35S promoter of cauliflower mosaic virus (CaMV). A DNA sequence inserted into the multiple cloning site of this vector's T-DNA should

therefore be efficiently transcribed in plants into which the T-DNA has been integrated.

5.3.2 Plant Material

5.3.2.a *Co-cultivation of seedling stem explants*

Allium cepa 'Pukekohe Longkeeper', 'Southport White Globe' and 'Hikeeper' seeds were surface-sterilized, with shaking, in a 30% v/v solution of commercial bleach (5.0% w/w sodium hypochlorite) plus a few drops of detergent, for 30-45 min. They were rinsed at least three times in sterile distilled water. Sterilized seeds were placed on 1/2x BDS salts (Dunstan and Short, 1977), pH 5.5, which was solidified with 0.7% agar, and were germinated in the light (cool, white fluorescent tubes, 30 μE $m^{-2}sec^{-1}$, 16 h daylength) at 20°C. Up to 300 five week-old seedlings were used for each co-cultivation experiment. Unbisected and longitudinally-bisected stem explants were prepared from these seedlings as described in section 3.3.5. All cuts were made in liquid log phase cultures of *Agrobacterium*, with each strain (except controls) harbouring one of the two binary vectors. These had been grown up in overnight shaking cultures of nutrient broth (Difco Bacto Laboratories, Detroit, USA) or LB medium (Miller, 1972), each containing 100 mgl^{-1} (for strains harbouring pKIWI110) or 20 mgl^{-1} (for strains harbouring pGA643) kanamycin sulphate. Cut tissue was left in bacterial cultures for approximately 1 minute, after which it was blotted dry on sterile filter paper. Explants were then placed on media for callus production (BDS medium with 1.5 mgl^{-1} picloram) or shoot production (BDS medium with no added PGRs). When media had cooled to approximately 37°C after autoclaving, 20 μM acetosyringone was added to half of all the BDS-based media used for co-cultivation (Sheikholeslam and Weeks, 1987). The pH of all acetosyringone- and non-acetosyringone-supplemented BDS media was adjusted to pH 5.5 prior to autoclaving. Two days later, co-cultivated explants were transferred to the callusing or shoot production media without acetosyringone, but with 250 mgl^{-1} cefotaxime (Claforan, Roussel Pharmaceuticals Pty. Ltd). One week later, explants were transferred to selection medium (30 mgl^{-1} G418, 250 mgl^{-1}

cefotaxime). Some of the explants which had been co-cultivated with strains of *Agrobacterium* harbouring pKIWI110, were placed onto callusing medium containing 50 nM chlorsulfuron instead of G418. These were also transferred onto chlorsulfuron-supplemented media for regeneration.

After approximately eight weeks, explants which had initiated growth on callusing medium with G418 or chlorsulfuron were transferred to regeneration medium (BDS medium, 1.6 mgl^{-1} BA, 0.4 mgl^{-1} picloram, pH 5.5), supplemented with 30 mgl^{-1} G418. One month later, the concentration of picloram in this medium was dropped to 0.1 mgl^{-1}. Lack of the usual regeneration response by callus, despite otherwise healthy growth, prompted the transfer of calli to BDS medium supplemented with 4 mgl^{-1} TDZ (section 3.3.2.b and 3.4.2) with 30 mgl^{-1} G418. After four weeks on the TDZ-supplemented medium, putatively transformed calli were taken off selection media and transferred to non-selective regeneration medium for 10-12 weeks. Following this, they were transferred back onto the G418-regeneration medium and then cultured for a further 10-12 weeks. Only those cultures surviving this second period of selection were assumed to be putative transformants.

5.3.2.b *Co-cultivation of callus*

Three-month-old 'PLK', 'ELK' and 'Hikeeper' callus was cut into small pieces in log phase cultures of *Agrobacterium* and co-cultivated on callusing (BDS, 1.5 mgl^{-1} picloram) or regeneration media (BDS, 1.6 mgl^{-1} BA, 0.4 mgl^{-1} picloram) for 2-3 days (see section 3.3.1 and 3.3.2 for detail on media). Half of these media contained 20 μM acetosyringone. Explants were then transferred to the callusing and regeneration media, containing 250 mgl^{-1} cefotaxime, but no acetosyringone. After a week they were transferred to these media containing cefotaxime and 30 mgl^{-1} G418.

5.3.2.c *Co-cultivation of protoplasts*

Protoplasts were prepared from callus cultures of 'PLK' as described in section

3.3.6. They were plated in liquid KM8p medium (Kao and Michayluk, 1975) with V-KM vitamins (Bokelman and Roest, 1983) substituted for KM8p vitamins, at a density of approximately 2.5 x 10^5 cells ml^{-1}. To each 500 μl of freshly isolated protoplasts in culture, 1 μl of log phase *Agrobacterium* culture was added. The *Agrobacterium* strains LBA4404, C58 and A4T, each harbouring the binary vector pKIWI110, were used in this experiment.

As *Agrobacterium* is known to bind to cell walls prior to infecting cells (Lippincott and Lippincott, 1980), protoplasts were also isolated, and cultured in the medium described above for four days without *Agrobacterium*, so that cell wall resynthesis could begin in this time. After four days in culture, the protoplasts were pelleted by gentle centrifugation (70g, 2 min) and the supernatant removed with a sterile pasteur pipette. Cells from *Agrobacterium* log phase cultures were pelleted by centrifuging for 15 min (3000g) at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 500 μl of fresh LB medium. Five μl of this mixture was added to protoplasts in 1 ml of fresh, sterile culture medium. Protoplasts were then incubated with *Agrobacterium* overnight. The following day cefotaxime was added to these cultures to a final concentration of 500 μg/ml. Two days later, the osmoticum of the culture medium was diluted slightly by the addition of 150 μl of KM8p (as above) with no glucose, sucrose or fructose, to 1 ml of medium containing the protoplasts (1:7.6 dilution). Selection of cells on antibiotics or chlorsulfuron was not carried out.

5.3.2.d *In vitro injection of twin scales*

Twin scales were cut from onion bulbs and surface-sterilized as described in section 3.3.4.a. Basal plate explants were injected in 1-4 places with log phase cultures of *Agrobacterium* using a sterile syringe with a fine needle. The needle entered the meristematic region of the basal plate. Injected basal plates were co-cultivated on onion shoot proliferation medium (pH 5.6) (section 3.3.4.b) with or without 20μM acetosyringone, for two days. They were then transferred to the same medium without acetosyringone but with 250 mgl^{-1} cefotaxime for 7 days, following which

they were transferred to selection medium (30 mgl^{-1} G418, 250 mgl^{-1} cefotaxime, pH 5.6). Cultures remained on this medium until live shoots could be separated from bleached and dying shoots. Live shoots attached to their immediate basal plate regions were separated from dying shoots at this stage, and were subcultured onto the same medium containing G418, to confirm stable resistance to this antibiotic. Ten weeks after the subculture, those plantlets still growing on G418 were taken off selection by transferral to the same medium lacking G418. They were cultured on this medium for up to 12 weeks. After this, these plantlets were transferred back onto G418-containing medium, on which they remained for at least 12 weeks. Only those shoots surviving the third period of selection were considered to be putative transformants, and at this stage, the number of shoots/plantlets were counted.

5.3.2.e *In vitro injection of basal meristems of tissue-cultured bulbils*

Split *in vitro* shoots were also used as explants for *in vitro* injections. Shoot cultures which had formed bulbils *in vitro* were split longitudinally (see section 3.3.4.b for details), and those which had not formed bulbils were left intact. Leaf growth was cut back to about 10mm above the basal plate region before the tissue was injected. Basal plates were injected with *Agrobacterium* in the same way as surface sterilized twin scales had been, except that only 1-2 injections per explant were carried out.

5.3.3 β-glucuronidase Assays

GUS assays were usually only carried out on tissue derived from inoculations with pKIWI110 binary vector strains which had survived the third period of selection on G418. Localization of GUS expression was assessed by the histochemical assay which uses 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) as a substrate (Jefferson, 1987). To suppress the endogenous GUS-like activity seen in control and 'transformed' onion tissue (section 5.4.5), 20% methanol was included in the reaction buffer (Kosugi *et al.*, 1990). Leaf, bulbil and root segments were incubated overnight with the reaction buffer containing 1 mM X-gluc, at 37°C. The following day, green tissue was soaked in 80% ethanol for at least 6 hours to decolour the

chlorophyll so that areas of blue precipitate could be seen.

5.3.4. NPTII Assays

Neomycin phosphotransferase II assays were usually only carried out on tissue derived from inoculations with either one of the binary vector strains which had survived the third period of selection on G418. NPTII enzyme was extracted from up to 700 mg of leaf tissue, and assayed using the method of McDonell *et al.*, (1987). Protein concentrations in these extracts were determined according to the Bradford (1976) technique as modified by Spector (1978). As NPTII activity was difficult to detect using the method of McDonell *et al.* (1987), protein in the supernatants was concentrated by precipitation with ammonium sulphate ($[NH_4]_2 SO_4$). This involved adding 561 gl^{-1} $(NH_4)_2SO_4$ to the supernatant (80% precipitation). Precipitated protein pellets were stored at 4°C. Prior to the assay each individual pellet was redissolved in 50 μl of fresh extraction buffer (McDonell *et al.*, 1987). Pellets were redissolved after the addition of extraction buffer by tapping the microfuge tube, vortexing, tapping again and then microfuging the mixture at 13 000 rpm for 5 minutes. The supernatant was saved and 15 μl was used for each assay.

Hairy roots of pea (*Pisum sativum* cv Pania) (Grant *et al.*, 1989) or turnip (*Brassica campestris* var *rapifera* cv Red Globe) (Christey and Sinclair, 1992), both of which had been transformed with A4T harbouring the pKIWI110 vector, were used as NPTII-positive controls. In addition, a positive control sample without substrate, and substrate with no plant extract were assayed. An onion sample was also assayed without substrate. These additional controls were carried out to check that a positive signal was not due to substrate degradation or non-specific binding of the ^{32}P to samples on the Whatman P 81 paper. The modified NPTII assay of Staebell *et al.*, (1990) was also performed on tissue of the same G418-resistant plantlets. To determine whether NPTII activity was localized to specific tissue types, leaves, bulblets and roots were assayed separately. To check if binary vector *Agrobacterium* strains were still living in the tissues, all plantlets which were to be used for the NPTII assay were smeared over an LB plate and dipped into LB broth, both

containing kanamycin (100 mgl^{-1} if inoculated with pKIWI110, 20 mgl^{-1} if inoculated with pGA643). No bacterial growth was seen on the solid or in the liquid LB medium.

5.3.5 DNA Extraction

The extraction of high molecular weight DNA from onion leaf and bulb tissue was initially carried out as described in the extraction method of Evans *et al.* (1983). As this method was very time consuming it was subsequently abandoned in favour of the quicker CTAB method of Lassner *et al.* (1989). Some minor modifications were made to this method by Dr J.E. Grant (pers. comm.). Instead of being squashed with sap extractor rollers, plant material (50-500 mg), which had been placed with 1.5 ml of extraction buffer in a small, seal-top plastic bag, was squashed with a wallpaper roller or rolling pin. The corner of the bag was cut and leaf/buffer liquid collected into a 1.5 ml microfuge tube. Subsequent steps followed the Lassner method. All DNA extracted, including controls, was quantified using OD_{260}/OD_{280} values as measured on a Philips UV/vis spectrophotometer (PU 8625). This quantitation assumes that an OD_{260} of 1 is equivalent to approximately 50 mgl^{-1} of double stranded DNA. The extinction ratios also provided an estimate of the purity of the nucleic acid being measured, with pure preparations of DNA and RNA having OD_{260}/OD_{280} of 1.8 and 2.0 respectively (Maniatis *et al.*, 1982).

5.3.6 Southern blot analyses

In total, 31 different Southern analyses were carried out. In these analyses, onion DNA was digested with *Hin*dIII, *Eco*RV, *Eco*RI or *Eco*RI/*Bam*HI. DNA for positive control samples was extracted from transgenic *Brassica* plants. Each plant had been transformed with one of two strains of *A. tumefaciens*, harbouring the binary vector pGV1047, pLN16 or pKIWI110, all of which carry the nptII gene. All *Brassica* transformations and DNA extractions were carried out by Dr Mary Christey, Crop & Food Research, Lincoln.

5.3.6.a *Digestion of DNA*

To initially establish which enzymes would digest onion DNA to a sufficient extent for Southern analyses, onion genomic DNA was at first incubated with a number of restriction enzymes including *Eco*RI, *Eco*RV, *Dra*I, *Bam*HI, *Pst*I and *Hin*dIII. Each reaction mix comprised up to 25 μg DNA, 12-18 units of enzyme, 4 μl of the 10x reaction buffer supplied with each enzyme, and sterile distilled water, if needed, to make the reaction volume up to a total of 40 μl. Digestions were incubated overnight at 37°C for approximately 16 h. Because digestion of onion DNA did not appear to go to completion with some enzymes (Plate 5.1a), it was decided that the reactions should be incubated for longer. After an overnight incubation, a further 8-12 units of enzyme was added to each digestion, and the reactions incubated for a further 4-5 hours. These digestions went to completion (Plate 5.1b). Reactions were stopped by the addition of 5 μl of sample buffer (Appendix VIII).

5.3.6.b *Electrophoresis*

Restriction fragments were separated by electrophoresis on a 0.9% agarose gel (BRL Ultra pure) in 1x Tris-Borate-EDTA (TBE) buffer (Maniatis *et al.*, 1982), usually run overnight at 35 V. All of the digestion mixture, i.e. up to 25 μg of DNA, was loaded per lane. After electrophoresis, the gel was rinsed in distilled water and stained with 0.5μgml^{-1} ethidium bromide for 20 min.

5.3.6.c *Blotting*

DNA was transferred from the agarose gel to a nylon membrane (Zeta-Probe, GT, Biorad) by capillary transfer using the method of Reed and Mann (1985).

5.3.6.d *Probe Isolation*

The plasmids pKIWI339 (constructed by Jeannette Keeling, Dept of Cell Biology, University of Auckland, New Zealand) and pKIWI101 (Janssen and Gardner, 1989)

were purified using the alkaline lysis plasmid minipreparation method (Sambrook *et al.*, 1989), as modified by Dr G. Timmerman (pers. comm.). The nptII coding region (1.25 kbp) excised from pKIWI339 was used to probe DNA extracted from G418-resistant plantlets. This probe was excised by digestion of pKIWI339 with *Eco*RI and *Xho*I for 1½ hours at 37°C, in a 10 μl reaction. A gus probe was also prepared to probe DNA from those plantlets which had shown GUS-positive responses. This probe was prepared from pKIWI101 by excising the 1.85 kbp gus coding region with *Eco*RI and *Bam*HI. Digestions were stopped by the addition of sample buffer. Plasmid fragments were separated on a 1% low melting point agarose (BRL Ultra pure) gel, cast on a microscope slide. The gel was electrophoresed at 80-85 V for 1-1½ h. After staining the gel with ethidium bromide, the fragment was excised in an agarose slice (Feinberg and Vogelstein, 1983). This DNA fragment could be used immediately or stored at 4°C for future use.

5.3.6.e *Labelling of probe*

Probes were radioactively labelled with ^{32}P by hexanucleotide random priming using the method of Feinberg and Vogelstein (1983). Specific activity of probes was measured with a geiger counter and sometimes quantified more accurately following TCA precipitation of probe DNA (Maniatis, 1982). When measured, specific activity was usually in the order of 0.5-2 x 10^9 cpm/μg probe. This compared favourably with 1 x 10^8 cpm/μg probe, recommended by the manufacturers of ZetaProbe membranes. As some problems were experienced with the above-described labelling reaction, probe DNA was subsequently labelled using a 'Multi-Prime' labelling kit (Amersham, U.K.)

5.3.6.f *Probe controls*

To check that nptII or gus would be detectable in the DNA of transformed onion tissue, known amounts of unlabelled nptII or gus probe were loaded into separate lanes on the same gels as the DNA from putative transformants and control DNA.

Unlabelled probe was combined with one of the control digests to see whether detection of probe DNA was affected by the presence of up to 25 μg onion DNA. Amounts of probe loaded ranged from 0.1 pg to 10 pg. Given that the onion genome has a size of 3.11×10^{10} bp per 2C nucleus (Arumuganathan and Earle, 1991), and that the nptII probe is 1.25 kbp in length, the amount of DNA equivalent to a single copy of nptII or gus could be calculated as follows.

As a percentage of the genome, the nptII probe was 4.02×10^{-6}:

$$\frac{1.25 \times 10^{3} \text{ bp}}{3.11 \times 10^{10} \text{ bp}} \times 100 = 4.02 \times 10^{-6} \%$$

If loading 10 μg of transformed onion DNA onto the gel, a single copy of the nptII 1.25 kbp coding region would be represented by 0.402 pg:

$$\frac{4.02 \times 10^{-6}}{100} \times 10 \times 10^{6} = 0.402 \text{ pg}$$

The gus probe is 1.85 kbp long. If loading a total of 10 μg of transformed onion DNA onto the gel, a single copy of gus would be represented by 0.595 pg DNA.

5.3.6.g *Prehybridization*

Freshly blotted, dried membranes were placed singly or doubly (DNA side out) into a container and washed in 2x SSC (preheated to 65°C) for 15 min. Membranes were then placed into a hybridization bag with 25 ml of prehybridization buffer (preheated to 65°C). The bag was sealed and membranes incubated 2-5 hours in a shaking waterbath at 65°C. Thirty ml of prehybridization buffer consisted of the following:

12 ml	25% dextran sulphate
9 ml	20x SSC (see Appendix VIII)
3 ml	10% SDS

900 μl	10 ng/ml herring sperm DNA (boiled for 5 min, then added to buffer at 65°C)
0.03g	$Na_2H_2P_2O_7$
5.1 ml	deionized, distilled H_2O

5.3.6.h *Hybridization*

After prehybridization, 5 ml of the prehybridization buffer to which denatured probe DNA (section 5.3.6.e) had been added, was poured into the membrane-prehybridization mix in each bag. Bags were resealed, placed in a 65°C shaking waterbath and membranes hybridized overnight for at least 16 hours.

5.3.6.i *Washing*

After hybridization, the probe mixture was poured off. Membranes were taken out of the hybridization bag and washed up to three times, each wash being for 30 min in a shaking waterbath at 65 °C, in successively more stringent SSC/SDS mixtures. These were, in order of washes, 2x SSC, 0.1% SDS; 1x SSC, 0.1% SDS; 0.1x SSC, 0.1% SDS.

5.3.6.j *Autoradiography*

If after the second wash (or occasionally after the first wash), little radioactivity was detected on the blot with a geiger counter, the third (or occasionally second) wash was omitted, and the blot autoradiographed at this stage. All washed membranes were exposed to Kodak X-Omat film at -70°C with intensifying screens, for periods of up to three weeks. The sizes of hybridization bands on the developed X-ray films were determined by plotting the mobility (cm from origin) of molecular weight markers (1 kbp ladder) against the known size of each marker as specified by the manufacturer (Gibco BRL, USA). These were plotted on log graph paper. Unknown band sizes were determined by interpolation.

5.3.6.k *Hybridization controls*

To check that the Southern hybridization techniques used were working effectively with onion DNA, control onion DNA was digested with *Eco*RI and blotted as described in section 5.3.6.c. This DNA was probed with a full length cDNA (approximately 1.7 kbp) encoding alliinase, an enzyme which is present in all onion tissues (Lancaster and Boland, 1990). The alliinase probe was produced by PCR from a cloned cDNA template, using Universal forward and reverse primers complementary to the flanking vector sequences. The alliinase probe was donated by Sonya Clark, and further details of its preparation can be found elsewhere (Clark *et al.*, 1993).

5.3.7 Polymerase chain reaction (PCR)

DNA was extracted from tissue of putatively transformed onions and controls by the method of Lassner (1989) and was analysed by PCR using protocols recommended by Perkin Elmer Cetus. Each reaction contained 0.1-0.3 μg DNA, 1 unit of *Taq* DNA polymerase (Boehringer Mannheim, Germany), reaction buffer (Boehringer Mannheim), 1 μM of each primer, deoxynucleotides (200 μM each of dATP, dGTP, dCTP and dTTP), with water to give a final volume of 25 μl per reaction. For the nptII primers, amplification conditions were 40 cycles of 94°C (1 min), 45°C (1 min) and 72°C (2 mins). The nptII primer sequences used were GAGGCTATTCGGCTAT and ATCTCGTGATGGCAGG. Using gus primers, amplification conditions were 33 cycles of 94°C (1 min), 45°C (1 min) and 72°C (2.5 min). The gus primer sequences used were TAATGCTCTACACCAC and CATACCTGTTCACCGA.

5.4. Results

5.4.1 Co-cultivation of excised seedling stems

5.4.1.a *Unbisected seedling stems on callusing medium*

When transferred to the first regeneration-selection medium (section 5.3.2.a), small amounts of callus were visible after two weeks on all co-cultivated seedling stems, including the controls. No responses, apart from further callusing, were seen on this or the second (reduced picloram) regeneration/selection medium (section 5.3.2.a). After being transferred to the TDZ-G418 regeneration-selection medium, approximately 20% of calli derived from all co-cultivated stems of the three cultivars, including controls, senesced within one month. On the surviving 80% of calli, several green, nodular areas were visible within approximately two weeks of transfer to this medium, and most of these subsequently produced shoot buds, roots and root-like structures. A further culture period of at least four weeks was required before chlorosis and senescence of control and untransformed callus began. Following chlorosis, the callus became watery and brown. By comparison, putatively transformed calli were creamy-yellow, usually with green nodular areas, and were fast-growing.

After a total of five months on the TDZ regeneration/selection medium, which included ten weeks off selection, only one plantlet (RC1) was regenerated, from 'PLK' seedling callus (Table 5.1). All other G418-tolerant calli and some control calli had taken up to four months to bleach and eventually die on the G418-supplemented media. From the 200 calli produced from seedling stems, no differences were observed between those which had been plated on acetosyringone- and non-acetosyringone-supplemented media (data not shown).

Seedling stem callus produced from two of the 'Hikeeper' explants co-cultivated with pKIWI110/LBA4404 grew and produced many roots on chlorsulfuron regeneration/selection medium (Plate 5.2). However, no plantlets or shoots could be regenerated from these roots.

5.4.1.b *Bisected seedling stems on shoot proliferation medium*

Once transferred to G418-supplemented medium, shoot and root production from bisected seedling stems was usually observed within two weeks. Some shoot-producing controls took up to four months to bleach and eventually die. After five months on selection medium, approximately 10% of the putative transformants were still alive. The presence of the nptII gene was not detected in the DNA of any of these plantlets following Southern analysis. After an eight week period without selection, followed by a further eight week period of selection, all plantlets had bleached and senesced.

5.4.2 Co-cultivation of established callus

After 3-6 months, some putatively transformed calli continued to grow slowly on the G418-supplemented medium. By this time most control calli were bleached and brown, excepting approximately 5% which had survived (data not shown). The presence of the nptII gene was not detected in DNA of any of the putatively transformed or control calli following Southern analysis. All co-cultivated calli were slow to die on G418-selection media.

5.4.3 Protoplast co-cultivation

Protoplasts derived from onion callus were denser and heavier than leaf protoplasts and did not generally float to the top of the 0.6M sucrose during the sucrose floatation step. To overcome this, more washing medium was added to the sucrose mixture and protoplasts were pelleted by centrifugation at (70g, 3 min). Co-cultivation with *Agrobacterium* did not appear to affect the viability of protoplasts. As with onion control protoplasts cultured without *Agrobacterium*, occasional cell divisions were seen, and cell walls were regenerated, but no difference was observed between *Agrobacterium* and non-*Agrobacterium* treatments.

5.4.4 *In vitro* injection of basal meristems

Providing that minimal damage was done to the basal meristem region during

injection with *Agrobacterium*, growth of new shoots was visible from most explants within 3-5 days of injection. When injected explants were first transferred to G418, shoot production was as prolific as that of uninoculated, unselected controls for 3-4 weeks. After this time some shoots began to show signs of chlorosis. Bleaching was usually first seen at the tip of the leaf. Within 8 weeks, some shoots were entirely bleached, while others remained green.

Growth of putative transformants on G418-supplemented medium was not usually as vigorous as growth of onion cultures on shoot proliferation medium lacking G418 (Plate 5.3a and b). Despite their slower growth on G418, these shoots continued to grow under selection pressure, well after all control shoots had bleached and senesced. Most of these shoots also produced roots while growing on G418. When taken off selective media, G418-resistant plantlets would grow more quickly than on media with G418. Only those plantlets or shoots which survived the third period of selection were considered to be putative transformants. Because adventitious and axillary shoots were often produced in close proximity to one another on injected basal plates, it was not always possible to tell if the G418-resistant clump of shoots had arisen from one or more than one of the original G418-resistant shoot clumps. (Table 5.1).

5.4.5 GUS histochemical assays

Initial histochemical GUS assays resulted in small amounts of activity being detected in all samples, including most controls. The inclusion of 20% methanol in the reaction buffer (Kosugi *et al.*, 1990) appeared to suppress this GUS or GUS-like activity, i.e. when methanol was present no blue precipitate was seen in the controls. Those plantlets assaying positively for the GUS gene (Table 5.2) showed only pale blue areas of activity (Plate 5.4). The root-forming callus growing on the chlorsulfuron-supplemented medium did not stain positively for GUS.

5.4.6 NPTII assays

Of the plantlets/shoots which showed resistance to G418 in culture, only one (TS3, see Table 5.2) which had been injected with pKIWI110/A4T, assayed positively for NPTII (Plate 5.5). This plantlet had been through one 12-week period of selection instead of the usual three, and extraction of the enzyme did not include the ammonium sulphate precipitation step. As many cultures were showing healthy growth on G418, it was thought that either the NPTII enzyme was being inhibited by a substance or substances present in the onion leaf extract, or that levels of protein in extracts were too low for the enzyme to be detected. To check for the presence of an inhibitor, extracts from onion leaves, bulblets and roots were added to the *Brassica* positive control samples, making up half of the total reaction volume. Addition of these extracts to positive controls did not inhibit NPTII activity. Protein concentrations of extracts were generally found to range from 600-2200 μg/ml, i.e., lack of activity in extracts was not due to a low concentration of protein.

Leaves, bulblets and roots which were assayed separately did not show any NPTII activity. Controls without substrate or without extract also did not assay positively for NPTII. No tissue assayed using the modified method of Staebell *et al.* (1990) was NPTII positive.

5.4.7 Southern analyses

Southern hybridization analyses of total onion DNA extracted from G418-resistant plantlets were performed to ascertain whether these tissues contained the expected T-DNA fragments.

Bands of the following sizes were expected: the restriction map of pKIWI110 predicts five internal fragments (1.3, 1.6, 2.1, 4.2, and 4.6 kbp) and two border fragments after digestion with *Eco*RI (Atkinson and Gardner, 1991), and two fragments at least 10.9 kbp and 7.1 kbp in length after digestion with *Hin*dIII (see Fig 5.1) (Janssen and Gardner, 1989). The 1.25 kbp nptII coding region probe should hybridize with a single 1.6 kbp band (the excised *nos*-nptII-*nos* chimeric gene) of the *Eco*RI digest and a larger band ($\geq$ 7.1 kbp) of the *Hin*dIII digest. DNA extracted from plants transformed with pGA643-*Agrobacterium* strains and probed with the

nptII coding region should show a band of at least 8.65 kbp in length after an *Eco*RI/*Bam*HI digest, a band of at least 3.3 kbp after digestion with *Hin*dIII and a band of at least 3.1 kbp after digestion with *Eco*RV (see Fig 5.2).

The lowest amount of unlabelled probe which could be consistently detected using the described probing method was 0.3 pg. Although the probe always hybridized with 0.3 pg or more of unlabelled probe, two bands were sometimes seen, instead of the single 1.25 kbp band expected (Plate 5.6a and b). Migration of unlabelled probe DNA was slightly retarded when co-electrophoresed with control onion DNA. This was evident as the nptII probe hybridized with a band or bands of slightly higher molecular weight in the lanes where control onion DNA had been co-loaded (Plate 5.6b, lane 1), as compared to the lanes containing only unlabelled probe DNA (Plate 5.6b, lanes 2-5). Non-specific hybridization of probe to high molecular weight onion control DNA was also observed (Plate 5.6b, lane 1).

Of the G418-resistant plantlets which were analysed, two contained fragments with homology to the nptII probe (Table 5.2). Some fragments were smaller than expected. One of these was the 1.0 kbp fragment from the *Eco*RI digest of RC1 (Plate 5.6b). The 1.5 kbp *Eco*RI fragment of RC1 which hybridized with the nptII probe was, however, close to the size predicted (1.6 kbp) (Plate 5.6a). The 1.5, 2.1 and 2.7 kbp fragments from the *Eco*RV digest of BP1 (Plate 5.7a) were smaller than expected, as was the 3.7 kbp fragment from the *Eco*RV/*Bam*HI digest of BP1 (Plate 5.7b) and the 1.7 kbp fragment from the *Hin*dIII digest of BP1 (Plate 5.7c). The intensities of all bands were low. By contrast, strong hybridization signals were seen in the DNA of *Brassica* positive controls which had been digested, blotted and probed in the same way as onion DNA (Plate 5.8). After probing DNA extracted from pKIWI110-inoculated, G418-resistant shoots with the 1.85 kbp gus probe, no hybridization of probe to DNA fragments was detected. The gus probe was, however, shown to hybridize with 0.3 pg or more of unlabelled gus probe.

5.4.8 Hybridization controls

After probing 'PLK' DNA with the alliinase cDNA clone, several positive

hybrization bands were visible (Plate 5.9). This established that the DNA hybridization method used was sensitive enough to detect the presence of a low copy number gene (Clark, 1993) in onion DNA.

5.4.9 Polymerase chain reaction

Bands of the sizes expected after PCR with the nptII and gus primers were not visible on the gel in which the PCR products were separated. Some bands of unexpected sizes were observed.

5.5. Discussion

Protocols for the *Agrobacterium*-mediated transformation of *Allium cepa* and evidence for such transformation have been presented in this chapter. To date, no reports of either have been found in the literature. Before considering stable transformation as the definite outcome of this work, however, the incidence of G418-resistant variants, escapes, transient expression of T-DNA genes and of *Agrobacterium* living in the inoculated tissue after co-cultivation, must be examined and dealt with. Resistance of plantlets to G418 was the first and only selection criterion (except in the small number of cases where chlorsulfuron was used as a selective agent). G418-resistance was observed more frequently than hybridization of DNA fragments to the nptII probe, or than GUS activity (in plants inoculated with strains harbouring pKIWI110). Hence, it might be assumed that some plantlets have been produced from variant or mutant $G418^R$ cells, or from escapes (see section 4.2 for more detail). The incidence of variants is a possibility in the case of BP2 and BI1 (Table 5.1 and 5.2), for which the only evidence of transformation is the continued growth of plantlets on G418, but not in the case of all other shoots or plantlets, for which there is further evidence of expression (TS1, TS2, TS3) or of integration (RC1, BP1) of T-DNA genes. Escapes would not have survived the second period of selection after being off selective media for 10-12 weeks.

The gus gene on the T-DNA of pKIWI110 has previously been shown to be transiently expressed for 1-4 days after inoculation with *Agrobacterium* (Janssen and Gardner, 1989). Hence it is possible that binary vector T-DNA transferred from *Agrobacterium* into the bulb injection sites, was being transiently expressed, but had not been stably inserted into the onion genome. For this reason, it was important to establish that the evidence for transformation was not just that of transient T-DNA expression. Growth of plantlets on G418 after at least three 10-12 week culture periods on selective medium, each of which had been interrupted by 10-12 week periods on G418-free medium, indicated that the expression of T-DNA genes was more than transient. Transient expression of nptII in plantlets may have been responsible for G418 resistance in the first period of selection, but was unlikely to be the explanation for growth of plantlets during the third or fourth selection period, as transient expression is not this long-lived (Janssen and Gardner, 1989).

The presence of agrobacteria living in or around the plant cells and thus being responsible for the G418-resistant and, in some cases, GUS-positive phenotype, can also be dismissed as a likely explanation for expression of these genes, as the nptII and gus genes in the expression vectors used were driven by *nos* and 35SCaMV promoters respectively. It has been shown that the *nos* gene is poorly expressed in bacteria, but actively transcribed upon transfer to the plant genome, the *nos* promoter being constitutively expressed in transformed tissues (An *et al.*, 1986). The chimeric gus gene has been constructed so that the 35SCaMV promoter is expressed in transformed plant tissues but not in bacterial cells (Jefferson, 1987, Janssen and Gardner, 1989). In addition, putatively transformed onion plants were shown to be free of surface-dwelling bacteria, as no bacterial growth was seen on LB media.

Unbisected seedling stems were the only explant type from which a G418-resistant plantlet (RC1) was regenerated via callus. Growth and regeneration from this callus on G418 provided evidence for expression of the nptII gene in RC1. The regeneration of one transformed plantlet out of 200 co-cultivated explants (0.5% regeneration) is not an unusually low frequency for a species which is recalcitrant to *Agrobacterium*-mediated transformation. Other workers (Schrammeijer *et al.*,

1990; Chee *et al.*, 1989)) have recorded similarly low ratios. Following co-cultivation of sunflower shoot meristems with *Agrobacterium*, Schrammeijer *et al.* (1990) found that from a total of 1500 explants, only two of the shoots produced were transformed. Similarly, Chee *et al.* (1989) recorded that only 0.7% of soybean plants grown from inoculated plumules, cotyledonary nodes and adjacent cotyledon tissues of 2000 germinating seeds were transformed. Given the sample size used in both of these studies, it appears that in future onion transformation experiments which use seedling stems as explants, at least 2000 explants of the same cultivar, tissue type and age should be co-cultivated with a single binary vector strain of *Agrobacterium*. Doing this, one would be able to reliably determine whether the 1:200 ratio of transformed:nontransformed plants was repeatable or whether this result was an anomaly. If the result were repeatable, conditions of co-cultivation, culture media, selection procedures and choice of *Agrobacterium* binary vector strain could then be adjusted to enhance transformation frequencies.

As RC1 was a small plantlet and did not provide sufficient tissue for an NPTII assay, no conclusions can be drawn about the expression of nptII, except that it was presumably being expressed in sufficient amounts to confer a G418-resistant phenotype. Southern blots confirmed that the nptII coding region was present in RC1. The DNA in both hydridizations was digested with *Eco*RI, which excises the 1.6 kbp *nos*-nptII-*nos* fragment of pKIWI110 T-DNA (Fig 5.1; Janssen and Gardner, 1989). This fragment would hybridize to the nptII probe regardless of whether it was integrated into the genome or not. However, since the other possible source of T-DNA i.e., agrobacteria living in the plant tissue, was not shown to be present, this result indicates that integration of the nptII gene into the genome of RC1 probably took place. As RC1 stopped growing and then senesced on G418-supplemented medium after approximately 9 months, further Southern analyses, and NPTII assays could not be carried out.

Established callus which had been inoculated with a range of *Agrobacterium*-binary vector strains senesced very slowly on kanamycin- and G418-supplemented media. It appears that co-cultivation of onion callus with an *Agrobacterium*-binary vector

strain containing the hpt gene, followed by selection on hygromycin-supplemented medium (section 4.4.3.c) may result in a better selection system and should be tried in future callus co-cultivation experiments.

It was thought that *in vitro* inoculations of basal plate tissues would result in a response to *Agrobacterium*, as this technique was effectively an *in vitro* form of the *in vivo* inoculations which induced tumour formation on bulbs (see Chapter 2; Dommisse *et al.*, 1990). Enzyme assay and Southern results for all plantlets putatively transformed via this inoculation method suggest that these plantlets could be chimeras. The following reasons offer support to this assumption: **firstly**, growth of 'transformed' plantlets on G418 was slower than on G418-free shoot proliferation medium (Plate 5.3). As soon as the plantlets were taken off their 10-12 week passage on G418, they grew rapidly, recovering to grow as quickly as those which had been off selection medium for weeks; **secondly**, with the exception of one plantlet, no G418-resistant plantlets assayed positively for NPTII activity. If the plantlets being assayed were chimeras, in which transformed cells contained only a single or low copy numbers of nptII, it is plausible that NPTII enzyme would be present in such low levels, that it would not be detected using this assay. The slow growth of the G418-resistant shoots and plantlets on G418 also implies that if present, the enzyme would only be there in small amounts. These two reasons may also be explained by poor nptII expression; **thirdly**, the bands produced in the Southern analyses were generally fainter than those of onion DNA laced with a single copy amount of nptII DNA, or than those resulting from onion DNA probed with alliinase. With the exception of DNA from one putatively transformed plantlet (BP1), fragments which hybridized with the nptII probe in the Southern analyses shown here are likely to have contained only a single copy of the nptII gene. This can be seen from the single hybridization bands seen in Plates 5.6a, 5.6b, 5.7b and 5.7c. The exception to this is seen in the blot containing DNA from BP1, which shows five hybridizing bands. This suggests that more than one copy of the insert is present in the genome of this transformant (see Plate 5.7a). This result was, however, not repeatable; **fourthly**, GUS activity was only detected in some of the shoots produced from the putative *Agrobacterium*/pKIWI110 transformants (Table

5.2). If each shoot was multiplied from a single GUS-positive transformed plantlet, one would expect GUS activity to be detectable in all or most of the shoots.

Single copies of nptII present in cells of chimeric transformants may have been effectively 'diluted' by the DNA of untransformed cells present in the extract. Given that the 2C onion genome is 32.2 pg, or 3.11 x 10^{10} bp (Arumuganathan and Earle, 1991), i.e., one of the largest known of the flowering plants, single copy genes may be difficult to detect. Single copy genes in chimeric tissue would be even more difficult to detect. It is possible that loading more than 25 μg of DNA from G418-resistant plantlets in each lane may have improved the detection of nptII by Southern analysis. A recent report of *Agrobacterium*-mediated transformation of wheat (Mooney *et al.*, 1991), mentions difficulties that were encountered with transformation and with detection of low copy number inserts in the large wheat genome (3.19 x 10^{10} bp, Arumuganathan and Earle, 1991). Mooney *et al.* (1991) found that foreign DNA was transferred to wheat via *Agrobacterium* at very low frequencies. Furthermore, when only single copies were transferred, the T-DNA had often undergone extensive rearrangements.

The low intensity of the band detected by the nptII probe in *Hin*dIII-digested DNA (thought to be a positive hybridization signal, Plate 5.7c), could probably have been increased by increasing the probe concentration 2-4 fold. Although the labelling reaction was not always optimal (section 5.3.6.e), the times when it was found to be optimal resulted in a high specific activity probe (0.5-2 x 10^9 cpm/μg). To obtain a probe of greater specific activity, PCR labelling of the probe could be performed. This was not done because of time limitations, but should be looked at in future onion transformation work.

A factor which should also be considered in these results is the condition of the nptII gene itself. Because nptII was the selectable marker widely used at the time this transformation work was initiated, all selection steps following co-cultivation were initially carried out on kanamycin, or, when kanamycin was shown to be ineffective as a selection agent (see section 4.4.1), on its analogue G418. The binary vector

first used, pKIWI110, was also being used at that time for *Agrobacterium*-mediated transformation experiments in other laboratories. However, it was observed by Drs Richard Gardner and Bart Janssen (Cellular and Molecular Biology Dept, Auckland University, N.Z.) that after co-cultivation with pKIWI110, and selection for stable transgenic events on kanamycin-supplemented media, the frequency of kanamycin-resistant calli or shoots was lower than had been obtained with other binary vectors. They found kanamycin resistance of transformants to be increased 5-10 fold when pGA643-based vectors were used instead of pKIWI110. The assumption made was that the pKIWI110 nptII gene may have been defective. After these findings, the use of pKIWI110 for onion transformation was stopped and pGA643 was used as a replacement binary vector.

As with the *Eco*RI-digested RC1 DNA, hybridization of the nptII probe to *Eco*RI/*Bam*HI fragments of BP1 DNA provided proof of T-DNA integration into the genome. *Eco*RI was one of a number of restriction enzymes initially used to digest onion DNA for Southern analyses with nptII. Subsequent to this result, DNA of G418-resistant plantlets which had been co-cultivated with *Agrobacterium* carrying pKIWI110 was mainly digested with *Hin*dIII, which restricts at a single site in the T-DNA. Similarly, DNA from G418-resistant plantlets which had been co-cultivated with strains carrying pGA643 was usually digested with *Eco*RV or *Hin*dIII, these two enzymes each restricting at single T-DNA sites (Fig 5.2).

The faint band seen in the *Hin*dIII digest of 'PLK' transformed with pGA643 (Plate 5.7c) shows that nptII DNA was probably integrated into the plant genome. Re-autoradiographing this blot resulted in a darker band, but background hybridization was also darker, which prevented a clear photograph from being taken. The 1.7 kbp band was smaller than the 3.3 kbp minimum size expected. This could have been be due to incomplete transfer or rearrangements of the T-DNA. Original analyses of T-DNA in plant tumour tissue showed that the T-DNA region was transferred intact into the plant genome (Lemmers *et al.*,1980), but subsequent work revealed the presence of truncated T-DNA insertions missing the right border region and some rearranged plant T-DNA inserts (De Beuckeleer *et al.*, 1981; Hepburn *et al.*,

1983; Ooms *et al.*, 1982). More recently, Deroles and Gardner (1988) found that at least 25% of transgenic petunias which had been selected for kanamycin resistance, were simple deleted derivatives that had lost one or both ends of the T-DNA. Approximately 20% of the T-DNAs of this 25% lacked the left border and at least this many lacked the right border. In addition, approximately 3% of plants contained grossly rearranged T-DNAs. This could also explain the smaller-than-expected bands seen in other digests (see Table 5.2). Although several *Hin*dIII digests of each G418-resistant onion plantlet were probed with nptII, the faint 1.7 kbp band from BP1 was the only band seen, suggesting that this may have been the most optimized Southern analysis performed with *Hin*dIII- digested DNA. This result was not repeatable.

Unexpected bands seen after Southern hybridizations, particularly as seen in Plates 5.6a, 5.7b and 5.7c, and the lane background seen in Plates 5.6a, 5.6b, 5.7b and 5.7c, may have been due to contaminated probe DNA. This could have happened if non-nptII DNA was present in the area immediately surrounding the 1.25 kbp nptII fragment isolated from a low melting-point agarose gel, and was therefore excised from the gel along with nptII DNA. As contaminating bands of the same size were present in digests from a number of different putatively transformed plants, each of which had been produced from separate inoculations, sometimes with different binary vectors, these bands could not be regarded as true positive hybridization signals. It should be noted that most of these blots were autoradiographed for 2-3 weeks, and when exposed to film for less than four days, no bands, except for those from hybridizations with *Brassica* positive control samples and the molecular weight markers, were visible.

The single NPTII-positive enzyme assay result obtained was not reproducible, despite repeated attempts and the modifications made to the method. In addition to the reasons already discussed, it is possible that the use of 80% ammonium sulphate to precipitate cellular proteins may have affected the NPTII activity. Similarly, it is also possible that insufficient protein pellet was redissolved after precipitation, as a good deal of the pellet was observed to be insoluble in the extraction buffer. This,

together with low copy number in chimeric tissue and/or poor expression of nptII, would more than account for the enzyme's activity not being detected.

The lack of detectable GUS activity in RC1 could be attributed to the particular shoot/s assayed having been regenerated from a cell/s which did not contain a copy of the gus gene. The gus gene is further from the pKIWI110 T-DNA's right border than nptII (see Fig 5.1). Because T-DNA transfer is initiated from the right border (Grant *et al.*, 1991) and the T-DNA is not always transferred in its entirety (De Beuckeleer *et al.*, 1981), it is likely that some G418-resistant cells contain the nptII gene, but not the gus or als genes. Atkinson and Gardner (1991) noticed that stable transgenic pepino plants arising from transformation with pKIWI110 had a low frequency of co-expression of kanamycin resistance with GUS activity and/or chlorsulfuron resistance. This was thought to be due to incomplete transfer of the large T-DNA of pKIWI110.

The failure to detect the nptII and gus genes in 'transformed' onion plants following PCR analysis may be due to the absence of these genes. It could be also be due to any number of factors being suboptimal for the PCR. This work was started late in the project, with insufficient time being available to optimize the PCR conditions. It is possible that the concentration of the integrated genes may have been too low to allow sufficient amplification for a band to be visualized on agarose gels. Unexpected bands may have been due to partial complementarity of primer sequences to some areas of onion DNA, resulting in non-specific product amplification (pers. comm. S.A. Clark). Adjusting factors such as the annealing temperatures and/or buffer conditions should be tried in future PCR analyses.

Of the several reasons why transformation has not been unequivocally demonstrated in onions, another one which must be considered is the strength of the promoters driving the nptII and gus genes. In both pKIWI110 and pGA643, the nptII gene is driven by a *nos* promoter. The production of large amounts of nopaline by *Agrobacterium*-induced tumours on onions (section 2.4; Dommisse *et al.*, 1990) is an indication that this T-DNA promoter can function in transformed onion tissue. It is

possible however, that the *nos* promoter region does not function optimally in onion tissue under *in vitro* culture conditions.

No clear evidence has emerged from these experiments to show that acetosyringone enhances the frequency of transformation. Although acetosyringone was added to some of the co-cultivation culture media, frequencies of G418-resistant shoots were similar on media with and without acetosyringone. This lack of noticeable response to acetosyringone was also seen in onion bulbs inoculated *in vivo* with *Agrobacterium* (section 2.4.3). Godwin *et al.* (1991), who co-cultivated onion seedling segments with virulent *Agrobacterium* strains, recorded no response to the bacterium, with or without 200μM acetosyringone in the culture media.

5.5.1 General comments and some suggestions for future work

Strains of *Agrobacterium* harbouring a binary vector which carried the hpt gene were not used in the transformation experiments carried out in the thesis. Work on onion responses to hygromycin was carried out towards the end of the project, not allowing time for such co-cultivations. Given that chlorosis and senescence of onion tissues occur more quickly in response to hygromycin than to G418 (section 4.4), future onion transformation experiments should involve the use of a binary vector with the hpt gene.

Chimeric transgenic plants resulting from inoculations with *Agrobacterium* have already been reported by other workers (Feldman and Marks, 1987; Chee *et al.*, 1989). Feldman and Marks (1987) co-cultivated imbibed seeds and Chee *et al.* (1989) injected germinating soybean seeds with *Agrobacterium*. Both of these transformation methods resulted in the production of chimeras. In such chimeras there are sectors of transformed and untransformed tissues. The transformed tissues may have arisen from one or more independent transformation events. Progeny from chimeras can be screened for completely transgenic plants which have resulted from individual transformation events (Grant *et al.*, 1991). 'Transformed' onion plants which are currently in tissue culture need to be transferred to the greenhouse

or field and allowed/induced to bulb, flower and set seed. Progeny from these chimeras should then be screened for G418-resistance at various stages of growth throughout their life cycle. Screening on G418-supplemented media during germination and growth of seedlings alone would not be sufficient, as germination is not effectively prevented by this antibiotic, and some 'PLK' seedlings appear to have an endogenous tolerance to G418 (section 4.4.2.a). Alternatively, the chimeras could be 'undone' by allowing shoots to callus and reselecting regenerated plants on G418. Time has not allowed for either approach to be carried out, as the cycle of a three month period of selection of transformed shoots, followed by 2-3 months off selective media needs to be repeated at least twice and preferably three times to confirm stable resistance of onion tissues to G418.

Table 5.1 G418-resistance of 'Pukekohe Longkeeper' and 'Early Longkeeper' shoots and plantlets regenerated from tissue inoculated with two *Agrobacterium* binary vector strains. All plants showing healthy growth on G418 were assayed for NPT II activity

Cultivar and tissue type	Inoculation technique	*Agrobacterium* binary vector strain	No. explants co-cultivated	No. shoots regenerated from explant[1]	Code name for plantlets/shoots[1]
'PLK', twin scales	*in vitro* injection, co-cultivation	pKIWI110/LBA4404	48	4	TS1
'PLK', excised seedling stems	co-cultivation	pKIWI110/LBA4404	200	1	RC1
'PLK', basal plates of *in vitro* shoots	*in vitro* injection, co-cultivation	pGA643/LBA4404	50	5	BP1
'PLK', twin scales	*in vitro* injection, co-cultivation	pKIWI110/C58	56	15	TS2
'PLK', twin scales	*in vitro* injection, co-cultivation	pKIWI110/A4T	25	0[2]	TS3
'PLK', basal plates of *in vitro* shoots	*in vitro* injection, co-cultivation	pGA643/A281	40	15	BP2
'ELK', bulblet of immature plant	*in vitro* injection, co-cultivation	pGA643/C58	30	2	BI1

[1] This number of shoots grew from what appeared to be a single shoot, but because adventitious and axillary shoots were often produced in close proximity to each other on injected basal plates, it was not always possible to tell if the G418-resistant clump of shoots was derived from one or more than one original G418-resistant shoot. For the purposes of this project, they are referred to as being derived from a single shoot.

[2] Shoots regenerated from twin scale explants did not survive the third selection period on G418.

Table 5.2 NPTII and GUS activity, and Southern analyses of G418-resistant shoots and plantlets.

Code name for plantlets/shoots	No. plantlets assaying positively for NPTII	% G418-resistant shoots showing GUS activity	Size of fragment expected to hybridize with the nptII probe	Size of band/s seen in Southern analyses (restriction enzyme)
TS1	0	75% (n=12)	as for RC1	-
RC1	not carried out (see text)	0% (n=5)	*Eco*RI: 1.6 kb	1.5 kb (*Eco*RI) 1.0 kb (*Eco*RI)
BP1	0	no gus gene on T-DNA	*Eco*RV: ≥3.1 kb EcoRI/*Bam*HI: ≥8.65 kb *Hin*dIII: ≥3.3 kb	1.5, 2.1, 2.7, 3.3, 4.5 kb (*Eco*RV) 3.7 kb (*Eco*RI, *Bam*HI) 1.7 kb (*Hin*dIII)
TS2	0	57% (n=7)	as for RC1	-
TS3	1	36%[1] (n=11)	as for RC1	-
BP2	0	no gus gene on T-DNA	as for BP1	-
BI1	0	no gus gene on T-DNA	as for BP1	-

[1] These assays were carried out before methanol was routinely used to inhibit intrinsic GUS-like activity. Plantlets did not survive the third passage on G418 and so could not be re-assayed for GUS activity.

Fig 5.1 A simplified diagram of the T-DNA region of the binary vector pKIWI110 (taken from Janssen and Gardner, 1989). This diagram is not to scale.

T-DNA of pKIWI110:

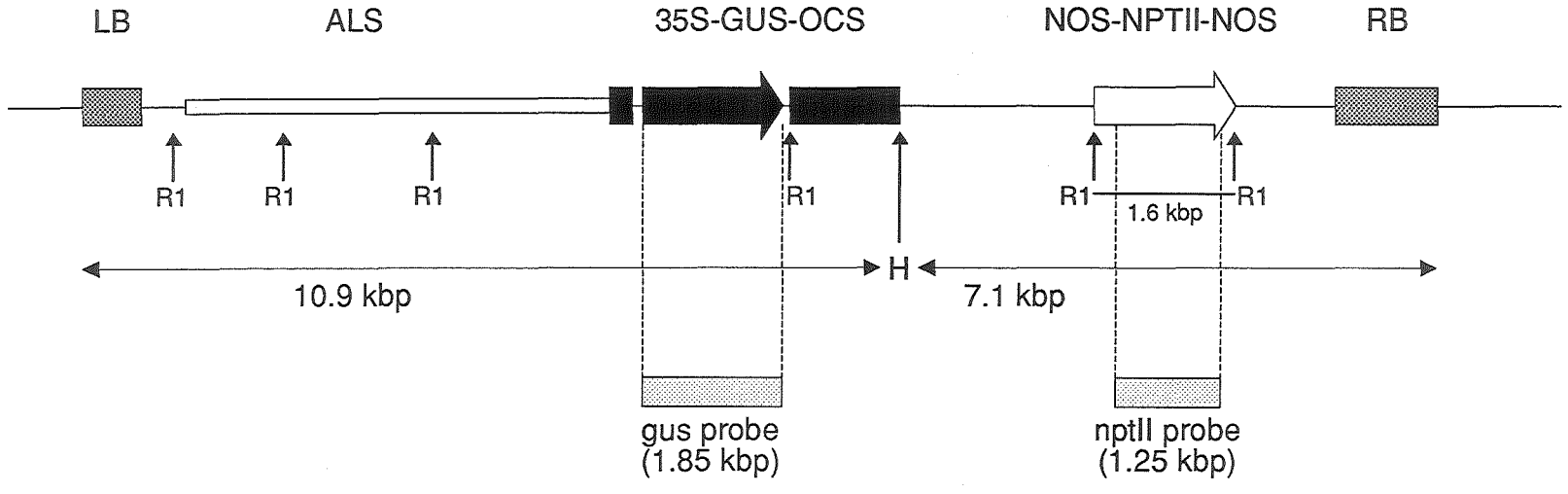


Fig 5.2 A simplified diagram of the T-DNA regions of the binary vector pGA643 (for details of vector construction see An *et al.*, 1988). This diagram is not to scale.

T-DNA of pGA643:

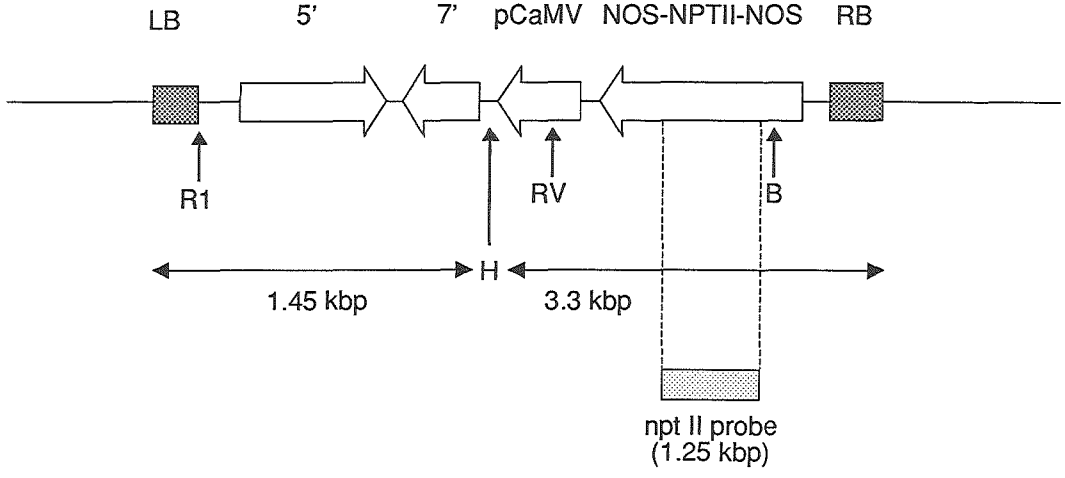
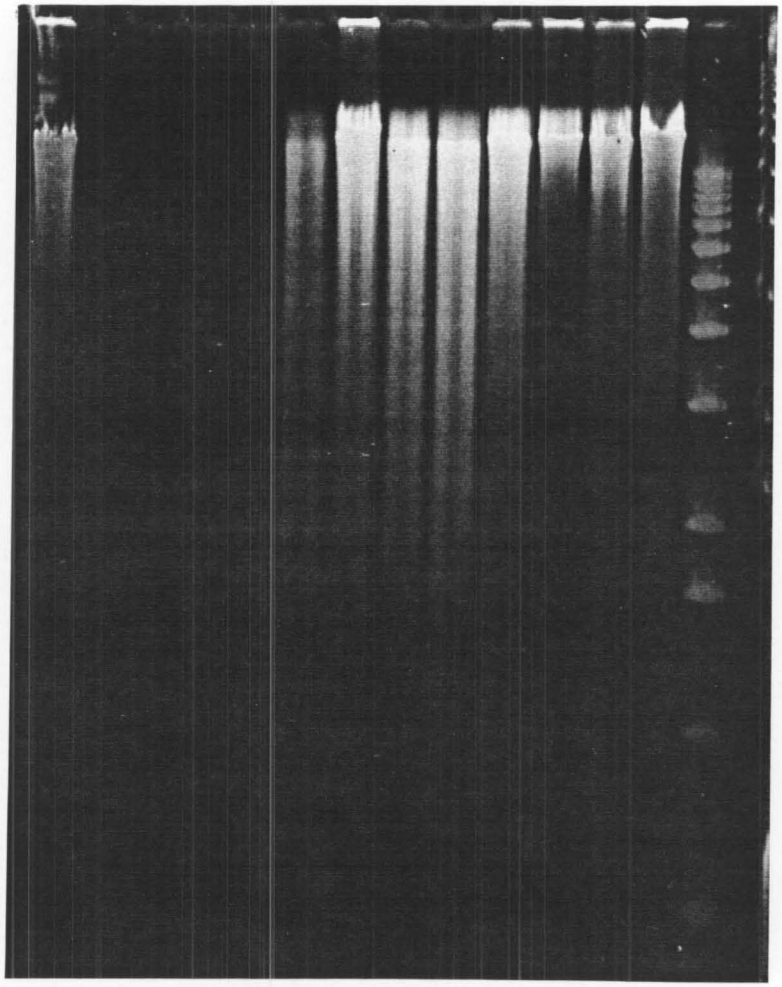


Plate 5.1 Incomplete (a) and complete (b) digestions of onion DNA after restriction with (a) *Eco*RI and (b) *Eco*RV

a



b

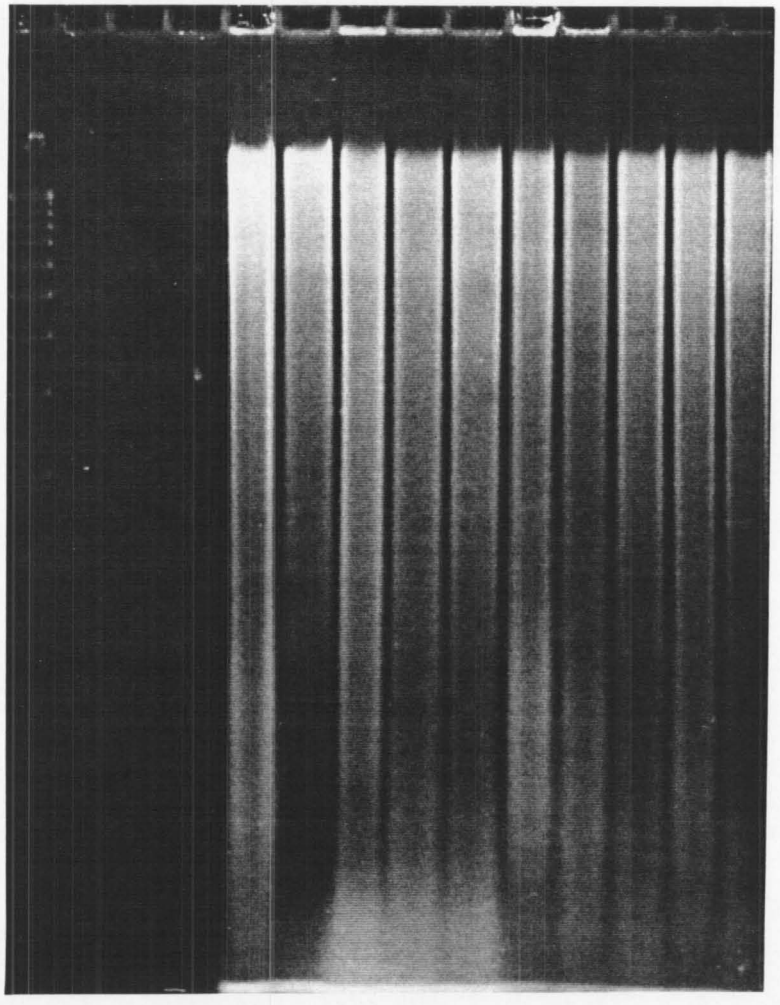


Plate 5.2 Roots produced by 'Hikeeper' seedling stem callus on regeneration medium (see text) with 50 nM chlorsulfuron (x5 actual size). Callus was produced from explants co-cultivated with LBA4404 harbouring the binary vector pKIWI110.

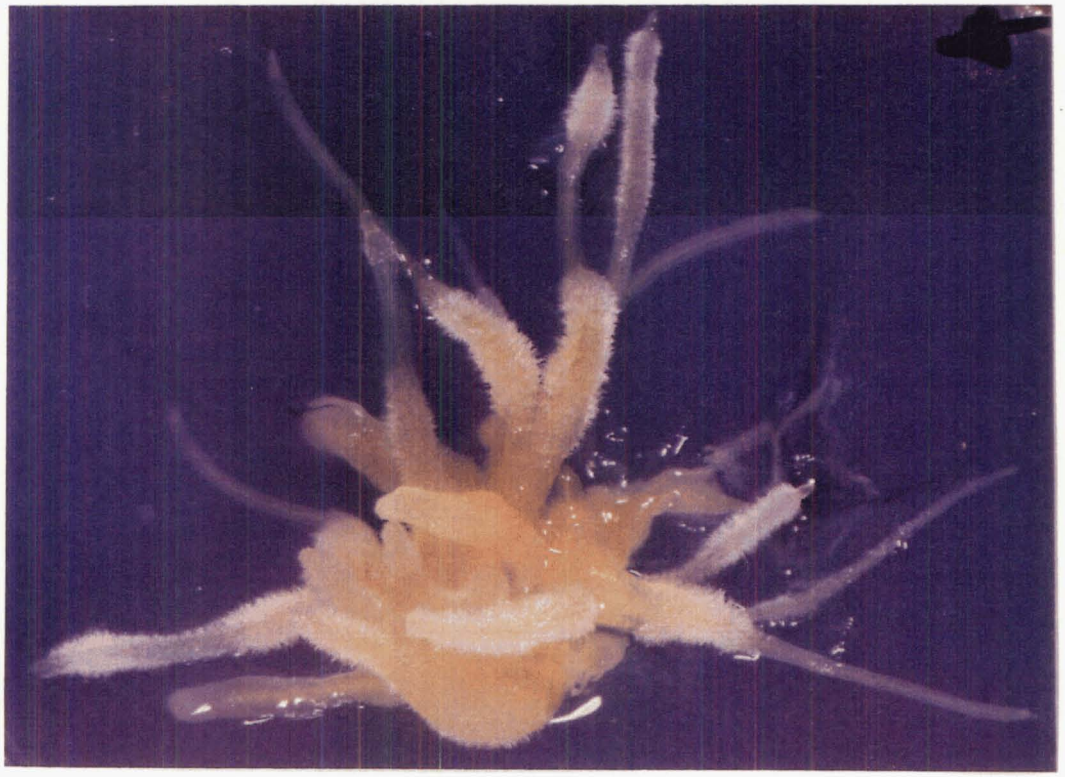


Plate 5.3 **(a)** Shoot cultures produced from basal plates of 'PLK' twin scales inoculated with (from left to right) (1) LBA4404 (control), and (2), (3) LBA4404 harbouring pKIWI110. Shoots in (1) and (2) have been on shoot proliferation-selection medium (30 mgl^{-1} G418, 250 mgl^{-1} cefotaxime) for 12 weeks, and shoots in (3) were on shoot proliferation medium with G418 for 10 weeks, after which they were transferred to medium without G418. These shoots have been off G418 for one week.

(b) Shoots produced from basal plates of 'PLK' *in vitro* shoots inoculated with LBA4404 containing pGA643. Shoots have been on shoot proliferation-selection medium (30 mgl^{-1} G418, 250 mgl^{-1} cefotaxime) for 8 weeks.

a

b

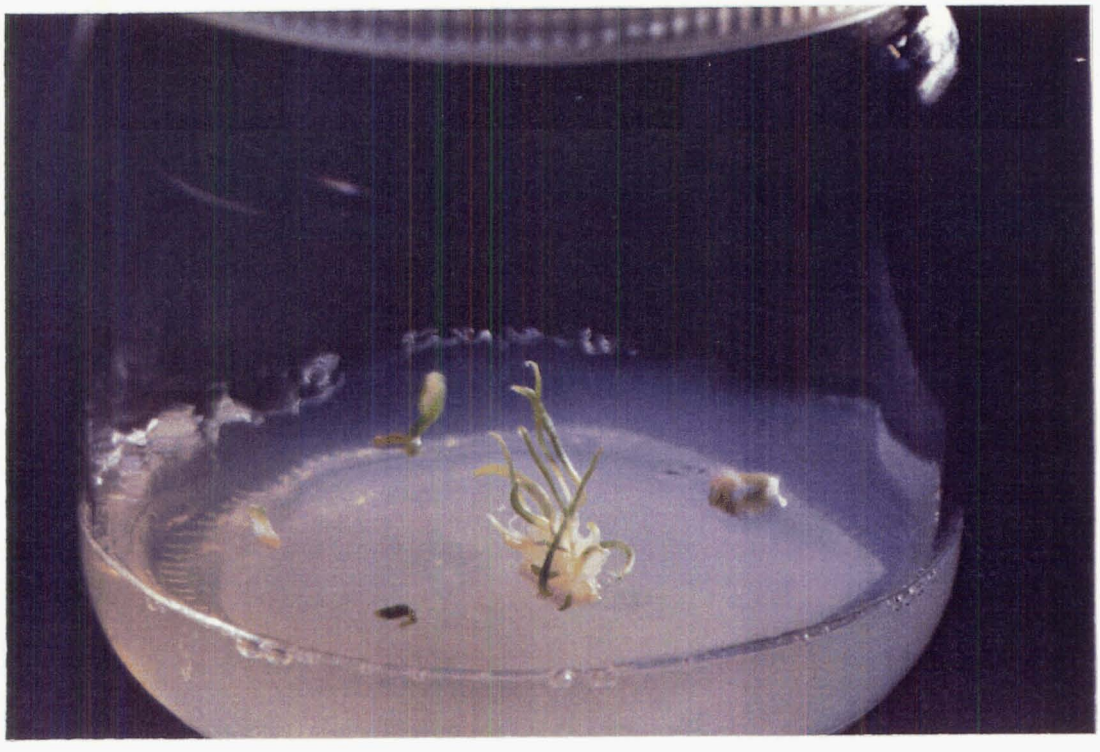
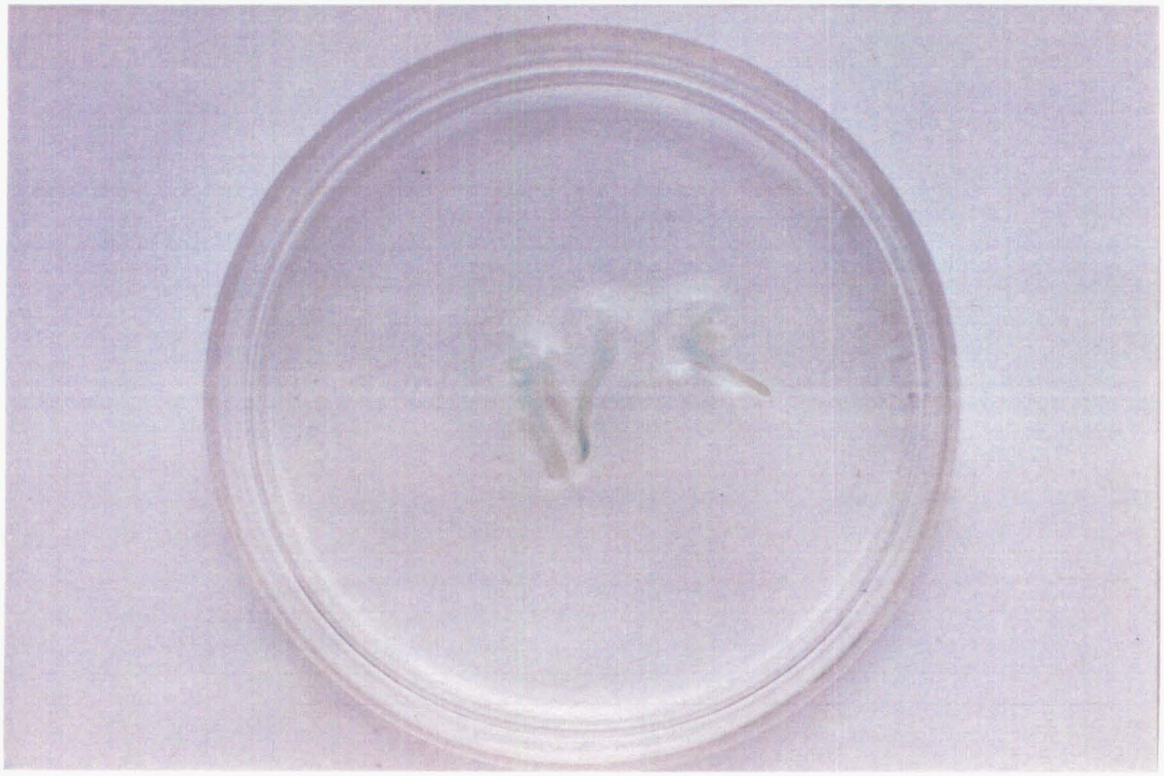


Plate 5.4 Areas of GUS activity in putatively transformed leaf tissue of 'Pukekohe Longkeeper' shoots. Leaves were produced from twin scales excised from bulbs which had been inoculated with *A.tumefaciens* strain C58 harbouring the binary vector pKIWI110.

Plate 5.5 NPTII assay: samples are (top left to right) (1) 'PLK' inoculated with LBA4404 (control), (2) 'PLK' inoculated with LBA4404/pKIWI110, (3) 'PLK' inoculated with C58/pKIWI110, (4) 'PLK' inoculated with HRI/pKIWI110, (5) 'PLK' inoculated with A4T/pKIWI110, (6) 'SWG' inoculated with A4T/pKIWI110 and (7) *Pisum sativum* cv Pania hairy roots produced after inoculation with A4T/pKIWI110 (positive control). Samples 1a-7a (2nd row left to right) are the same as those in 1-7, but with no substrate.



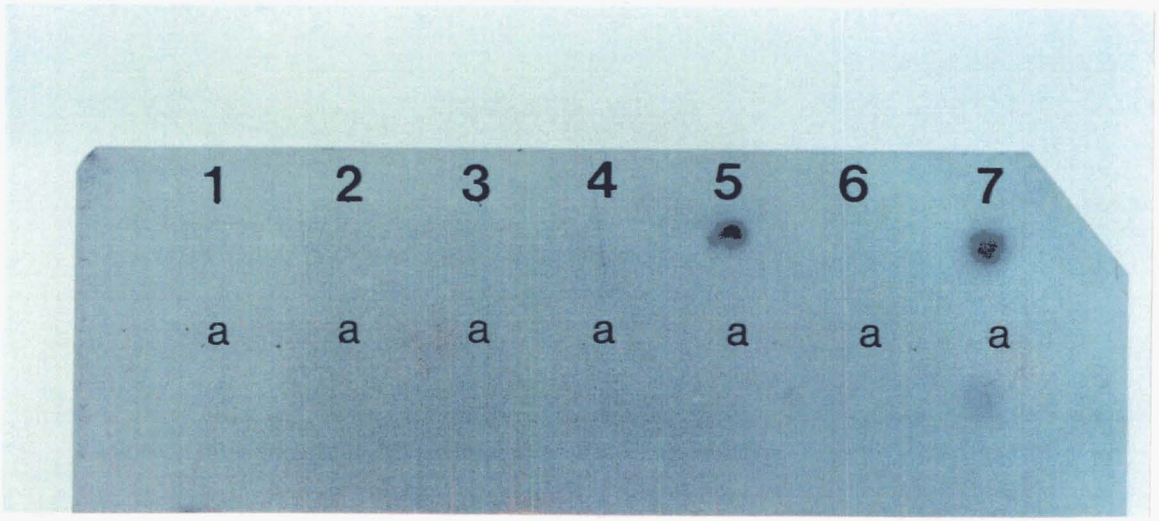
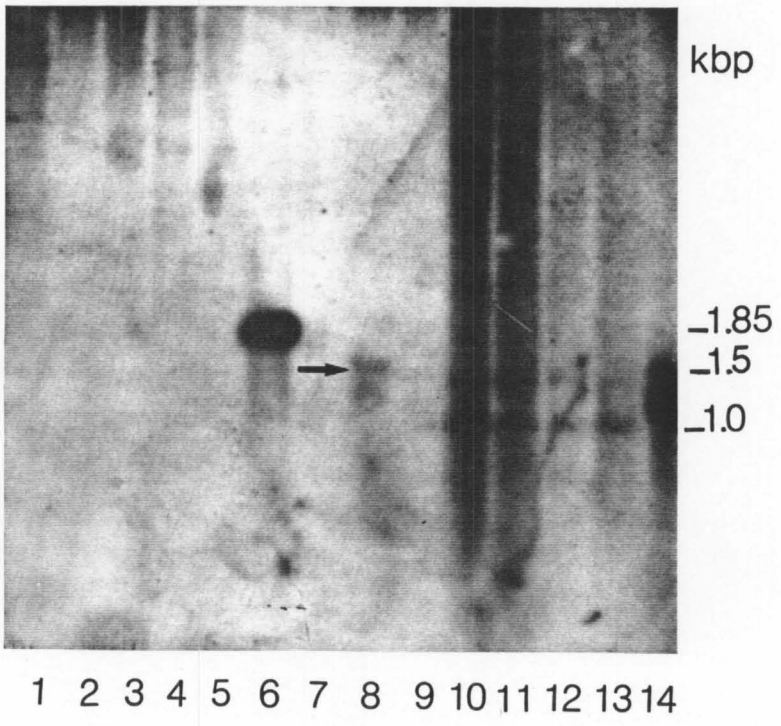

1 2 3 4 5 6 7
a a a a a a a

Plate 5.6 Southern analyses of DNA from G418-resistant shoots and plantlets. In both **(a)** and **(b)**, 'PLK' DNA was digested with *Eco*RI and probed with the 1.25 kbp nptII probe.

(a) Lanes (from left to right) contain DNA from (1) and (2) separate shoots of TS3, (3) a shoot from TS1, (4) young leaves and (5) older leaves from the same shoot of RC1, (6) positive control i.e. transformed *Brassica campestris* cv Red Globe plantlet (see Plate 5.8 (f) for details), (7) blank lane, (8) a separate shoot of RC1 (the 1.5 kbp band is arrowed), (9) onion control shoots , (10) 10 pg probe, (11) 5 pg probe, (12) 1 pg probe, (13) 0.5 pg probe and (14) 0.5 pg probe co-electrophoresed with untransformed onion DNA.

(b) Lanes (from left to right) contain DNA from (1) onion control digest with added nptII probe DNA (0.5 pg), (2) 0.5 pg probe DNA, (3) 1 pg probe DNA, (4) 5 pg probe DNA, (5) 10 pg probe DNA, (6) RC1 (the 1.0 kbp band is arrowed), (7) TS1, (8) and (9) separate shoots of TS2 and (10) onion control shoots.

5.6 a



5.6 b

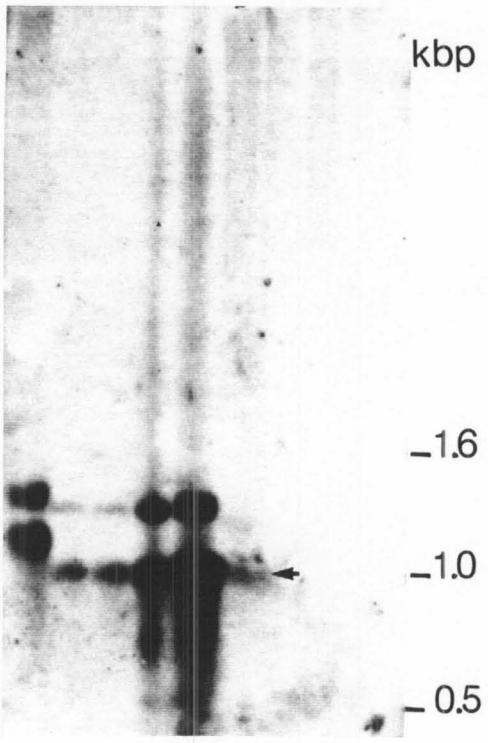
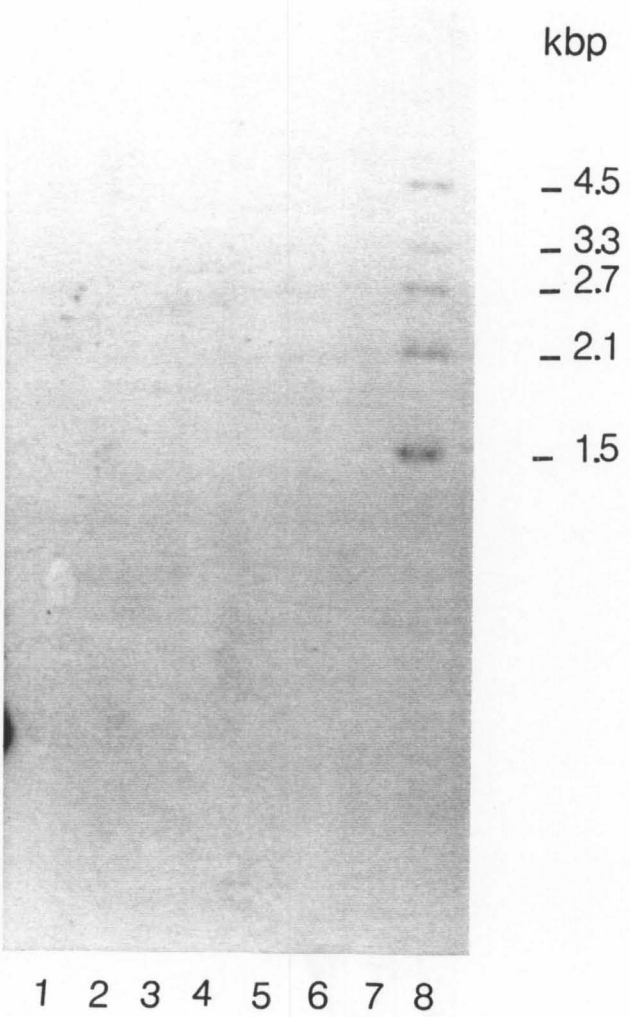


Plate 5.7 Southern analyses of DNA from G418-resistant shoots and plantlets, probed with the 1.25 kbp nptII probe. DNA was digested with **(a)** *Eco*RV, **(b)** EcoRV/*Bam*HI and **(c)** *Hin*dIII (see overleaf for **5.7 (c)**).

(a) Lanes (from left to right) contain DNA from (1) onion control shoots, (2) BP2, four separate plantlets of TS2 (3), (4), (5) and (6), (7) onion control shoots and (8) BP1.

(b) Lanes (from left to right) contain DNA from (1) onion control plantlet, (2) roots of RC1, (3) leaves of RC1, shoots of TS1 (4), BP1 (5), BI1 (6) and TS2 (7). The 3.7 kbp band in the digest of BP1 is arrowed.

5.7 a



5.7 b

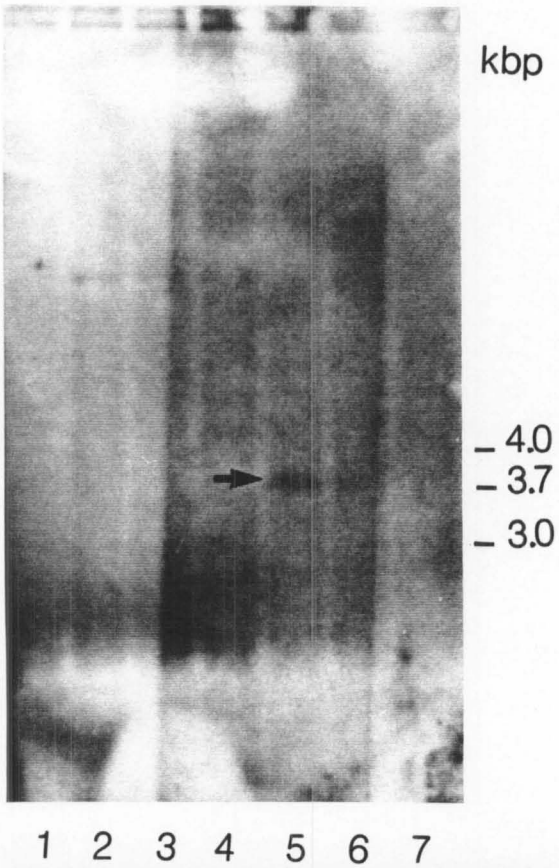
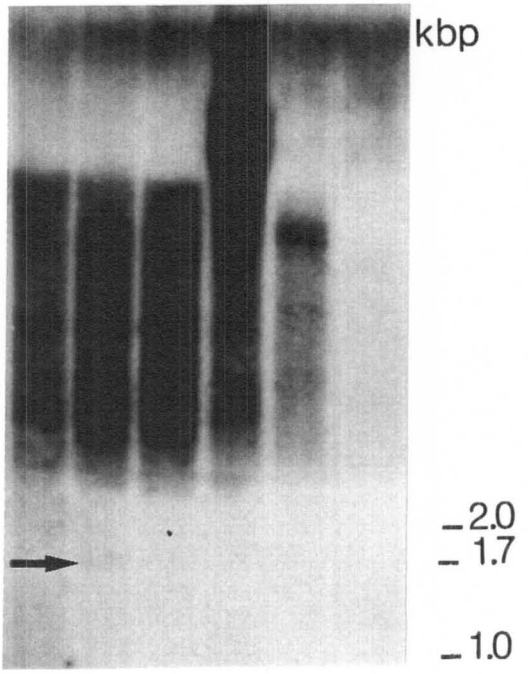


Plate 5.7 (continued)

(c) Lanes (from left to right) contain DNA from (1) TS1, (2) BP1, (3) BI1, (4) TS2, (5) the transformed *Brassica* positive control (see Plate 5.6a) and (6) onion control plantlet. The faint 1.7 kbp band thought to be a positive hybridization signal is arrowed.

Plate 5.8 Southern blot showing hybridization of DNA from transgenic *Brassica* plantlets (see section 5.3.6 for details). DNA samples were digested with *Eco*RV and probed with the 1.25 kbp nptII probe. Lanes (from left to right) contain DNA from (a) transformed rapid cycling (RC) *Brassica oleracea* plantlets regenerated after co-cultivation with EHA101/pGV1047, (b) another regenerant from the same co-cultivation as (a), (c) RC *B. oleracea* control inoculation (co-cultivated with LBA4404), (d) transformed giant rape (*B. napus*) plantlets regenerated from hairy roots (co-cultivated with A4T/pLN16) and (e) transformed turnip (*B. campestris* cv Red Globe) plantlets regenerated from hairy roots (co-cultivated with A4T/pKIWI110). DNA from 'transformed' and control onions has been electrophoresed in lanes (f) to (l). Although very faint bands are visible in lanes (g) and (h), these bands were also visible in the control DNA lanes when the blot was exposed to X-ray film for longer.

5.7 c



5.8

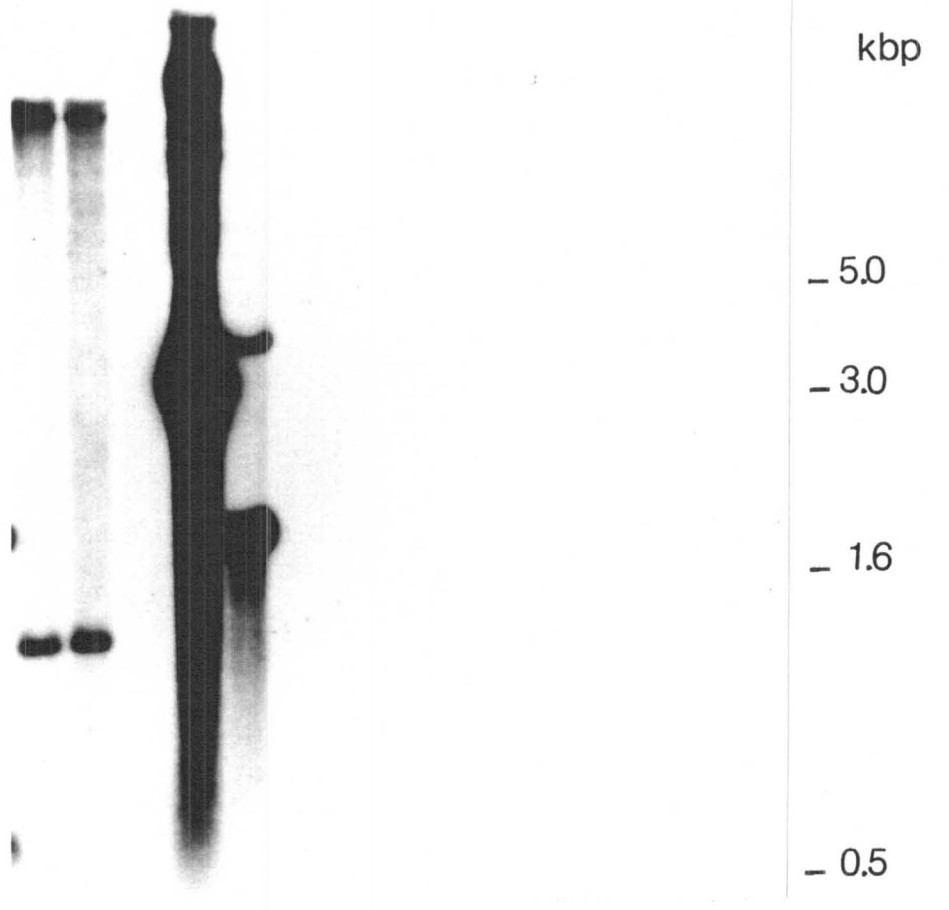
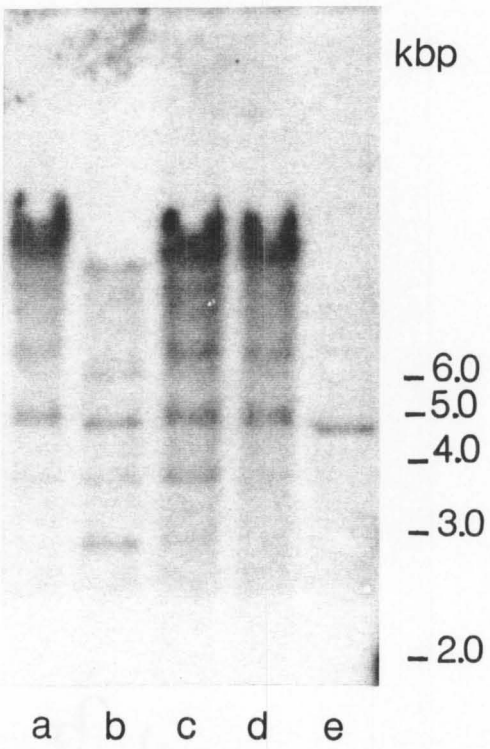


Plate 5.9 Southern analysis of untransformed 'Pukekohe Longkeeper' DNA probed with a 1.7 kbp cDNA clone of alliinase. DNA was digested with *Eco*RI. Lanes (from left to right) contain DNA from *in vitro* shoot cultures 1 (a), 2 (b), 3 (c), 4 (d) and 5 (e).

5.9



Chapter 6: General discussion

6.1 Summary of findings of this thesis

This study has conclusively shown that *Allium cepa* is a host for *Agrobacterium tumefaciens*, *A. rhizogenes* and *A. rubi* infection (Chapter 2). Nopaline- and octopine-producing tumours were formed on bulbs of 'Pukekohe Longkeeper' and/or 'Early Longkeeper' onions, after inoculation with 25 virulent *Agrobacterium* strains. The production of tumours in response to inoculations with 72% of the wild-type strains used, indicated that 'PLK' and 'ELK' bulb tissue had a wide range of strains to which it was susceptible. However, tumour responses were tissue- and genotype-specific. Of all the plant regions inoculated, only the area of the bulb surrounding the basal meristem consistently produced tumours in response to *Agrobacterium* inoculations. This response was thought to be due to the proximity of the cells to the basal meristem. A genotype-specific response to *Agrobacterium* was demonstrated, with only two of the four cultivars inoculated showing susceptibility to *Agrobacterium* infection. After excision and surface sterilization, some of the 'PLK' tumours produced roots in sterile culture. Despite several attempts at regeneration on a range of media, plantlets could not be regenerated from *in vitro*-cultured tumours or from roots produced by these tumours.

Following numerous attempts at co-cultivation with armed and disarmed strains of *Agrobacterium* harbouring the binary vectors pKIWI110 or pGA643, a single transformed 'Pukekohe Longkeeper' plant was regenerated (Chapter 5). This originated from callus derived from onion seedling stem tissue which had been co-cultivated with LBA4404 harbouring pKIWI110. Growth of this plantlet on G418-supplemented media over an extended time period, and positive Southern hybridization results provided evidence for transformation. In addition, 41 of the axillary or adventitious

shoots, growing from basal plate regions which had been injected with *Agrobacterium* harbouring the above binary vectors, showed resistance to G418 over an extended time period, when grown in *in vitro* culture. Most of the shoots regenerated roots in culture on G418. DNA from five of these shoots was shown by Southern analysis to hybridize with an nptII probe, indicating the presence of this gene in plantlets thought to be transformed. Nineteen of the 41 G418-resistant shoots produced from inoculated basal plate tissue were produced after inoculations with *Agrobacterium* harbouring the binary vector pKIWI110. Of these, 75% of shoots/plantlets produced following inoculation with *Agrobacterium* strain LBA4404/pKIWI110 showed GUS activity, and 57% of shoots/plantlets produced following inoculation with strain C58/pKIWI110 showed GUS activity. Due to the evidence for transformation of all shoots/plantlets produced by the *in vitro*-injection technique, which included slow growth on G418-supplemented media, faint bands in Southern hybridizations and irregular results in GUS histochemical assays, it was thought that plantlets/shoots produced by this technique were chimeric.

Tissue culture experiments carried out with eight onion genotypes showed that all of these genotypes could be clonally multiplied from bulb basal plate tissue and that four of them were regenerable from callus of seedling origin (Chapter 3). Subsequently, a technique was developed that enabled multiplication of plantlets directly from the longitudinally-bisected stems of onion seedlings. These explants would be ideally suited to *Agrobacterium* co-cultivation experiments, but transformed plantlets or shoots have not so far been produced via this technique. Culture of onion protoplasts in a range of media resulted in first cell divisions, formation of new cell walls and cell budding, but no further progress was made.

Work done following these transformation experiments showed that hygromycin was more toxic to all *in vitro*-grown onion tissues tested than G418 or kanamycin (Chapter 4). Earlier work had shown that onion tissues grown on kanamycin-supplemented media responded only slowly to kanamycin, with chlorosis of shoot tissue not being evident until twelve weeks after transfer of shoots to the antibiotic. G418 was shown to be more toxic than kanamycin to shoot tissue and also to most other onion

tissues surveyed. For this reason, explants which had been co-cultivated with *Agrobacterium* harbouring pKIWI110 or pGA643, both of which carried the nptII gene as their antibiotic selectable marker, were screened on G418-supplemented media. The herbicide chlorsulfuron was also shown to be toxic enough to onion tissues to be an effective selective agent.

6.2 A general discussion of results

In a recent review on gene transfer methods, Potrykus (1991) states that proof of integrative transformation of plants requires the following:

1. controls for treatment and analysis,
2. a tight correlation between treatment and predicted results,
3. a tight correlation between physical (e.g. Southern blot) and phenotypic (e.g. enzyme assay) data,
4. complete Southern analysis containing (a) the predicted signals in high molecular weight DNA, including hybrid fragments between host DNA and foreign gene, and the presence of the complete gene, and (b) evidence for the absence of contaminating DNA fragments or identification of such fragments,
5. data that allow discrimination between false positives and correct transformants in the evaluation of the phenotypic evidence,
6. correlation of the physical and phenotypic evidence with transmission to sexual offspring and
7. molecular and genetic analysis of offspring populations.

Although some of the above types of evidence for *Agrobacterium*-mediated transformation of onions have been presented in this thesis, further evidence is still needed to provide unequivocal proof of transformation. Limitations to the transfer of foreign genes to onions via *Agrobacterium* have become apparent. Transform-

ation at the cellular level was more readily achieved following *in vivo* inoculations of onion bulbs which incited opine-positive tumour responses, than with *in vitro*-inoculated explants which were co-cultivated with *Agrobacterium*. The production of several G418-resistant shoots from inoculated basal plates has limitations because of the likely chimeric nature of the putatively transformed plantlets produced, and the resulting difficulty in obtaining reproducible positive DNA hybridization results.

The traditional lack of susceptibility of most monocotyledonous plants to *Agrobacterium* has in the past been attributed to a number of factors, some of which have since been shown to be incorrect. These include the theory that *Agrobacterium* fails to bind to monocotyledonous cell walls in the same way that it does to dicotyledonous walls (Rao *et al.*, 1982). Since this theory was first put forward, scanning electron microscope studies have shown the attachment of *Agrobacterium* cells to cell walls of wheat embryos (Mooney and Goodwin, 1991) and to cell wall-regenerated protoplasts of rice and asparagus mesophyll cells (Terouchi *et al.*, 1990). It has more recently been hypothesized that only plants and tissues with a pronounced wound response will develop larger populations of wound-adjacent cells competent for efficient transformation, i.e. dicots that have so far been untransformable, probably do not show the appropriate wound response (Potrykus, 1990). Transformation of 'monocots' as a group appears therefore to be of no particular importance in the context of difficulties experienced in trying to transform recalcitrant plants, e.g. cereals, as the reason cereals are difficult to transform is not because they are monocots, but rather because they do not show a wound response. According to this theory, monocots with a wound response are probably as readily transformed as dicots with a wound response. Similarly, dicots without a wound response are as difficult to transform as cereals.

By the use of cladistic methods to analyse anatomical, biochemical and molecular data, taxonomists have shown that the Class *Monocotyledones* is probably monophyletic, i.e., it comprises all the descendants of a single ancestral species. By contrast, the dicotyledons are now thought of as a paraphyletic group i.e., they do not comprise all the descendants of a single ancestral species and have therefore been

grouped together under what is for some genera and families, an artificial classification system not based on common ancestry (Dahlgren and Bremer, 1985). Although monocotyledons are probably a monophyletic group, it is possible that the more basal groups within the monocotyledon monophyletic group (clade), may share the same response as the dicotyledons to a pathogen such as *Agrobacterium*. The response typical of cereals and grasses (Poaceae or Gramineae family) is probably a derived response within the monocotyledons, and not characteristic of the entire group.

The basal clades of monocotyledons may thus share numerous ancestral (plesiomorphic) biochemical or molecular characteristics (e.g. plant cell wall structure, DNA sequences) with dicotyledons rather than with more derived monocotyledons such as grasses. Such plesiomorphic characteristics are of no value in assessing evolutionary relationships, but it is important to consider them in the context of this study. Analysis of onion cell walls has shown that (4')-linked galactans and a substituted xyloglycan are major cell wall components (Mankarios *et al.*, 1980). Xyloglucan showed structural features in common with the xyloglucans of dicotyledonous plants, indicating that onions resemble dicotyledonous plant species more than they resemble those belonging to the Poaceae in their cell wall composition (Redgwell and Selvendran, 1986). Jarvis *et al.*, (1988) also found that monocots which did not belong to the graminoids (Poaceae, Cyperaceae, Juncaceae and Restionaceae) had high galacturon and other pectin contents, comparable with those of dicots. Furthermore, Bacic *et al.*, (1988) postulated that the monocots that lacked wall-bound ferulic acid (including onions) had walls with similar compositions to those of dicotyledons. A recent study of codon usage, i.e. selective and non-random use of synonymous codons by an organism to encode amino acids in the genes for its proteins, has resulted in similar conclusions (Campbell and Gowri, 1989). These workers found that two classes of genes could be recognized in monocots. One set of monocot genes used codons similar to those used in dicots, while genes from plants in the Poaceae family were highly biased towards codons ending with the bases cytidine (C) or guanosine (G). Another study of restriction fragment maps of the chloroplast DNA (cpDNA) of four lilioid

monocots, including *Allium cepa*, showed that chloroplast genome arrangements of these monocots were more typical of dicots than they were of species belonging to the Poaceae (Chase and Palmer, 1989). This adds support to the above explanation (Potrykus, 1990) of dicot/monocot susceptibility to *Agrobacterium*.

It appears, from work carried out in this thesis, that onions fall into the group of monocots described by Potrykus (1990) as having a distinct wound response and therefore being potentially as 'transformable' as dicotyledonous plants which show wound responses. Some progress has been made towards the transformation of onions, but barriers preventing the production of transformed plants consisting entirely of transformed cells, i.e. not chimeric, still need to be overcome.

6.3 Suggested improvements to the protocol for *Agrobacterium*-mediated transformation of onions

Throughout the course of this study, it has become apparent that further work should be done on some parameters which facilitate *Agrobacterium*-mediated transformation of onions. One such parameter is the use of a good selectable marker. The work done on dose responses to antibiotics with a range of onion tissue types showed that hygromycin is clearly more toxic to all types of tissue tested than kanamycin or G418, indicating that hpt appears to be a good selectable marker for use in onion transformation experiments.

In future experiments, different binary vectors, each having the hpt gene controlled by a different promoter, e.g. *nos*, 35S, and the 2' transcript of the octopine T_R-DNA (Velten *et al.*, 1984; Dekeyser *et al.*, 1989), should be used in *Agrobacterium*-mediated transformation of onions. Enhancing the frequency of onion transformation may however require the use of a promoter which is known to function optimally in onion tissue. One such promoter is the region of DNA which drives the onion gene

alliinase. Alliinase is expressed in a range of onion (and other *Allium*) tissues (Lancaster and Boland, 1990). This promoter region is currently being isolated by Brent Gilpin (Crop & Food Research, Lincoln, New Zealand). Once isolated, the alliinase promoter region attached to an hpt gene may be an effective chimeric gene to use in onion transformation experiments. Another promoter which should be considered for use in onion transformation experiments is the recombinant promoter region, *pEmu*. This promoter has been constructed from four discrete elements, and has been shown to give a high level of gene expression in five different graminaceous monocots (Last *et al.*, 1991). Finding a promoter which has optimal activity in onions, using sufficiently large numbers of seedling stem explants in co-cultivation experiments and making appropriate modifications to the co-cultivation procedure could well enhance *Agrobacterium*-mediated transformation of onions.

Factors which have been discussed as reasons for sub-optimal transformation of onion tissues should also include appropriateness of tissue types. In addition, co-cultivation conditions required for the successful transfer of T-DNA to the DNA of inoculated onion tissues may also not have been optimal. The transformation frequencies of other monocotyledonous species have been found to be very dependent on the type of explant which was co-cultivated. Kuehnle and Sugii (1991) found that after inoculation with *Agrobacterium*, *in vitro* tumours were induced on etiolated internode tissue of *Anthurium andraenum* (Araceae family) 16 times more frequently than on green leaf tissue, and ten times more frequently than on petiole explants. With the exception of dark-grown callus and dark-grown seedlings, etiolated onion tissue was not generally included in co-cultivation or *in vitro* injection experiments. In future, experiments involving the co-cultivation of etiolated onion tissue with *Agrobacterium* should be carried out.

6.4 Agronomic applications of *Agrobacterium*-mediated transformation of onions

When *Agrobacterium*-mediated transformation of onion has been optimized, and the frequency of transformation events is more predictable, attempts to transfer genes of agronomic importance into this crop can commence. The *Agrobacterium* vector system is already being extensively used to transfer desirable traits to other crop plants and is also used for the study of gene function in plants. Genes which have to date been transferred to plants include those affecting traits as diverse as herbicide tolerance (De Block *et al.*, 1987), virus resistance (Powell-Abel *et al.*, 1986), altered flower colour (van der Krol *et al.*, 1988), lengthened shelf life (of tomato) (Smith *et al.*, 1988), male sterility (Mariani *et al.*, 1990), cold tolerance (Hightower *et al.*, 1991), altered source-sink relationships (von Schwaenen *et al.*, 1990), altered starch composition (Visser *et al.*, 1991), starch derivatization to cyclodextrin (Oakes *et al.*, 1991) and resistance to pathogenic bacteria (Anzai *et al.*, 1989). Extensive field testing is currently being carried out on these modified crops, and it will not be long before some of them are available to the consumer.

6.4.1 Engineering genes conferring resistance to bacterial and fungal diseases into *Allium cepa*

One of the principal cost factors currently affecting onion production in New Zealand is disease control. Damage to onions caused by pathogens, particularly those causing bacterial soft rot and onion white rot, results in significant losses of export earnings each year. Bacterial soft rot (also termed 'vinegar rot') is one of the most complex and intractable problems faced by New Zealand onion growers, as no effective control measures are known (Hale *et al.*, 1992). It can affect onions at any stage of growth or storage. Several species of soil-borne bacteria, including *Pseudomonas marginalis*, *P. viridiflava*, *P. gladioli* pv. *alliicola* and *Erwinia carotovora* can cause soft rot. The presence of diseased onion bulbs led to a decline in exports to Japan from 78.7% of total exports in 1984 to 20.7% in 1987. Diseased bulbs were most commonly infected with bacterial soft rot. The fungus *Sclerotium cepivorum* Berk. causes onion white rot. This fungus is present in all onion growing areas of New

Zealand and has been of increasing concern in the Pukekohe district, the area of New Zealand in which all exported onions and 75% of the national crop are grown (Hale *et al.*, 1992). Onion white rot is a disease which most commonly affects plants during growth but it can continue as bulb rot during storage. Other pathogens which can be responsible for considerable crop losses during growth or storage include *Botrytis allii* Munn (neck rot) and other *Botrytis* species which cause only minor damage, *Sclerotium rolfsii*, *Peronospora destructor* (Berk.) Fr (downy mildew), *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker and Larsen (pink root rot), *Urocystis cepulae* (smut) and a variety of fungi which cause pre- and post-emergence damping off of onion seedlings.

Resistance to bacterial and fungal pathogens would be a desirable characteristic to engineer into onions, boosting New Zealand's export earnings from the crop. Durable resistance, i.e. resistance that remains effective while the cultivar possessing it is widely cultivated in an environment that favours the disease, may be conditioned by a single gene or by many genes (Johnson, 1983; Sharp, 1983). Such resistance has already been introduced into tobacco via *Agrobacterium*, conferring resistance to wildfire disease which is caused by *Pseudomonas syringae* pv *tabaci* (Anzai *et al.*, 1989). The introduced acetyltranferase gene (*ttr*) encoded the enzyme responsible for detoxifying tabtoxin, the toxin produced by *P. syringae* pv *tabaci*. This strategy could be widely applied to combat other bacterial diseases, including those affecting onions, which produce pathogenic toxins. One drawback of this strategy is that a number of genes must be introduced into a single plant to detoxify the variety of toxins produced by an array of different pathogens. In contrast to insect-tolerant plants engineered with a bacterial insect-toxin, microbial-tolerant plants have also been obtained by using insect-derived lytic peptides. This area of research has been reviewed in detail by Destefano-Beltran *et al.*, (1990), but briefly, it involves the incorporation of genes encoding insect-derived potent anti-microbial proteins to increase resistance of plants to bacterial and fungal pathogens. In future experiments, this strategy could also be employed to combat onion diseases, using *Agrobacterium*-mediated transformation.

Resistance to fungal pathogens in plants is conferred by several classes of defence-related genes. Expression of these genes is triggered by pathogen attack, environmental stress and biotic or abiotic elicitors (Chakravorty and Scott, 1991). Such defence-related genes are grouped into three classes by Bowles (1990). These include those encoding (1) glycoproteins, glycine-rich proteins and enzymes involved in strengthening and repairing the cell walls, (2) antimicrobial proteins and (3) pathogenesis-related (PR) proteins, the function of which is still unclear. Introduction of these genes into plants is still in the experimental stages in most cases, but progress has been made towards the production of transgenic plants which are resistant to fungal infection. As well as increasing the yield of healthy bulbs, *Agrobacterium*-mediated transfer of a gene which, when expressed, protects onions from fungal disease damage, would offer onion growers a less labour-intensive and less toxic alternative to spraying with fungicides.

6.4.2 Engineering genes conferring resistance to viral diseases into *Allium cepa*

The most important *Allium* virus which affects onions is the onion yellow dwarf virus (OYDV). This virus is sap-transmitted, principally by aphids (Walkey, 1990). OYDV is not seed-transmitted, however, so that onions grown from seed, as is the practice in New Zealand onion-growing areas, are initially free of virus infection when first established in the field.

So far, plant genetic engineering approaches used to produce virus-resistant plants have mostly involved the integration of viral cDNA sequences into plant genomes (Timmerman, 1991). Powell-Abel *et al.* (1986) first used *Agrobacterium* to introduce a cloned cDNA of the coat protein (CP) gene of tobacco mosaic virus (TMV) into tobacco. Transgenic tobacco plants expressed the CP gene which resulted in a virus-resistant phenotype. Since then, a non-structural coding region from TMV has also been used to produce virus-resistant plants (Golemboski *et al.*, 1990). Other strategies for producing virus-resistant plants include the introduction of satellite RNAs (reviewed in Timmerman, 1991) or antisense RNAs which are complementary to viral positive-sense sequences (Cuozzo *et al.*, 1988). Genetically modified *Agro-*

bacterium could in future be used as a vector to introduce viral DNA or RNA to onions. Expression of these viral nucleic acids could confer resistance to OYDV and other less important viral diseases.

6.4.3 Engineering genes conferring resistance to insect pests into *Allium cepa*

The production of onions in New Zealand can also be affected by insect pests, some of which include the shallot aphid (*Myzus ascalonicus* Doncaster), onion thrips (*Thrips tabaci* Lindeman), cutworm (*Agrotis ipsilon* Hufnagel), small narcissus fly (*Eumerus strigatus* Fallen), large narcissus fly (*Merodon equestris* F.), leek or shallot fly (*Delia platura* Meigen), the beetle species *Agrypnus variabilis* Cand., the weevil species *Ctenicera strangulata* White, red spider mites (*Tetranychus cinnabarinus*) and brown wheat mites (*Petrobia latens*) (Soni and Ellis, 1990).

The major emphasis in developing insect-resistance of plants has been on the development of plants containing a protein isolated from the soil-dwelling bacterium *Bacillus thurigiensis* (B.t.). Protein crystal spore preparations of B.t. have been used as commercial preparations for several years and it is estimated that over 2000 tonnes of this preparation have been applied to crops worldwide with no undesirable effect (Dunwell and Paul, 1990). To date, several genes encoding insecticidal B.t. proteins have been inserted into plants and have been shown to protect plants against lepidopteran insect pests (moths and butterflies) under field conditions (Delannay *et al.*, 1989). More recently, additional genes which are specifically active against coleoptera (beetles), diptera (flies) and nematodes have been isolated (Dunwell and Paul, 1990). As well as plants being transformed with the B.t. gene, at least one plant species has been transformed with a gene driven by the wound-inducible promoter of proteinase inhibitor II K (*pin2*) (Thornburg *et al.*, 1990). In response to insect feeding, plants transformed with *pin2*-controlled chimeric genes can specifically direct the synthesis of insecticidal proteins. With the *Agrobacterium* transformation of onions in place, genes conferring resistance to insect pests may possibly be engineered into the New Zealand cultivars of onion which are susceptible to the above-mentioned insects.

6.4.4 Engineering genes conferring resistance/tolerance to herbicides into *Allium cepa*

Another biological factor which is responsible for significantly reduced yields of onion crops is that of weed competition. Due to their slow germination, slow growth rate and upright, narrow-leaved habit, onions are very sensitive to early season weed competition. Weeds competing with onion seedlings for light, CO_2, O_2, water and mineral nutrients can significantly reduce the final crop yield even if for a short period early in the growth/development of seedlings (Bleasdale, 1959). The 'critical period' of competition has been identified by Hewson and Roberts (1971) as the period during the development of the third true leaf.

The regeneration of tobacco plants resistant/tolerant to the herbicide glyphosate (the active ingredient of the Dupont herbicide Roundup) was the first example of such resistance being engineered into a plant species via *Agrobacterium* (Comai *et al.*, 1985). Since then, genes coding for herbicide resistance functions have been introduced into some of the major crops. Three types of approaches tried have been successful. These are (i) overexpression of the sensitive target enzyme of the herbicide, (ii) altering target sites on enzymes via point mutations and (iii) incorporating a gene for an enzyme that inactivates the herbicide. As an example of (i), glyphosate-tolerant canola and soybean have been produced. These plants, which show a tolerance to Roundup, were transformed with DNA coding for over-production of herbicide-resistant analogues of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the target of Roundup activity (Shah *et al.*, 1986). Resistance to sulfonylurea compounds, the active ingredients of Glean and Oust, has been conferred by a mutant acetolactate synthase (als) gene (see section 4.2) introduced into canola and cotton, thus demonstrating the approach outlined in (ii) (Haughn *et al.*, 1988). Resistance to gluphosinate, the active ingredient in Basta (De Block *et al.*, 1987) and bromoxynil (Stalker *et al.*, 1988) has been conferred via *Agrobacterium*-mediated transformation by approach (iii) above, i.e. introducing bacterial genes encoding enzymes that inactivate the herbicides by acetylation or

nitrile hydrolysis respectively. Gluphosinate-tolerant corn, soybean and canola, and bromoxynil-tolerant cotton have all proved themselves in field trials. One of the three described approaches should also be eventually feasible for onions. Herbicide-resistant seedlings could in this way survive applications of herbicide that are toxic to competing weeds.

Herbicide resistance also offers some new applications to hybrid seed production in onions where there are problems with environmental effects on expression and stability of male sterility. In the past, fertility restoration in certain male-sterile lines of onions has made it uneconomical to produce hybrid seed from otherwise excellent crosses (Grant, 1983). If hybrid seed is produced from a 'male' parent that is homozygous for a single dominant herbicide-resistance gene and from a 'female' (i.e. male-sterile) parent, all true hybrid seed harvested from the 'female' parent would be heterozygous for resistance to the herbicide. Any plants arising from contaminating pollen can be simply eliminated by their sensitivity to the herbicide (Conner *et al.*, 1991a).

6.4.5 Manipulation of onion flavour

In addition to the need for disease-, insect- and herbicide-resistant onion breeding lines, there is also a demand, particularly from Japanese consumers, for onions exported from New Zealand to have a milder, less pungent flavour (Wood, 1986). A cDNA copy of the gene alliinase, a vacuolar enzyme which catalyzes the ultimate reaction responsible for flavour, odour and pungency of onions and other alliums, has recently been cloned and partially sequenced (Clark *et al.*, 1993). It is possible that onions, when transformed with a reverse copy of the alliinase gene, may show inhibited expression of alliinase, due to the presence of transcribed antisense RNA. Such inhibition of a gene's expression by an antisense copy of the same gene has already been achieved in transgenic tomatoes which were stably transformed with antisense DNA of the developmentally-regulated gene polygalacturonase (Smith *et al.*, 1988). Plants expressing the antisense RNA showed a striking inhibition of polygalacturonase activity. Similarly, it may be possible that onions transformed

with an antisense copy of alliinase will, due to the inhibition of alliinase expression, have a milder flavour.

6.5 Conclusion

In conclusion, this thesis reports that:

- *Allium cepa* is a host for *Agrobacterium tumefaciens*, *A.rhizogenes* and *A.rubi* as is evidenced by the production of opine-positive tumours,

- the New Zealand-bred onion cultivars 'Pukekohe Longkeeper' and 'Early Longkeeper' were susceptible to *Agrobacterium* infection, whereas other cultivars showed no evidence of susceptibility to this bacterium, despite the distinct wound responses which were recorded in non-susceptible cultivars,

- two of the trialled protocols were shown to be successful in achieving production of putatively transformed onion plantlets,

- an onion plantlet putatively transformed via *A. tumefaciens* was regenerated from callus, and a number of putatively transformed chimeric plantlets or shoots were produced from basal plates injected with strains of *Agrobacterium* harbouring the binary vectors pKIWI110 or pGA643,

- hygromycin appears to be the most suitable antibiotic for use in onion transformation experiments *and*

- onion seedling stems and twin scales or split *in vitro* shoots are the

most suitable explants for use in onion transformation experiments.

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Appendix I Formulations of basal media used for protoplast culture.

	Concentrations of basal salts			
	MS	8p	V-KM	BDS
Macro	(mM)			
NH_4NO_3	20.6	7.49	-	4.0
KNO_3	18.8	18.7	14.6	25.02
$CaCl_2.2H_2O$	3.0	4.08	5.0	1.02
$MgSO_4.7H_2O$	1.5	1.22	4.0	1.00
KH_2PO_4	1.25	1.25	0.5	-
$NH_4H_2PO_4$	-	-	-	2.0
$(NH_4)_2SO_4$	-	-	-	1.01
$NaH_2PO_4.H_2O$	-	-	-	1.04
KCl	-	4.02	-	-
$(NH_4)_2SO_4$	-	-	-	1.01
Micro	(μM)			
KI	5	4.5	4.5	4.52
H_3BO_3	100	48	48	49
$MnSO_4.4H_2O$	100	59	59	45
$ZnSO_4.7H_2O$	30	7	7	6.95
$Na_2MoO_4.2H_2O$	1.0	1	1	1.03
$CuSO_4.5H_2O$	0.1	0.1	0.1	0.1
$CoCl_2.2H_2O$	0.1	0.1	0.1	0.105
Iron	(μM)			
Na_2EDTA	100	-	100	100
$FeSO_4.7H_2O$	100	-	100	100
Sequestrene^{R}330 Fe	-	28 mgl^{-1}	-	-

Appendix II Responses of *in vitro*-cultured 'Pukekohe Longkeeper' tissues to kanamycin

Kanamycin concentration (mgl^{-1})	% germination		Average weight of callus produced by seedlings (g)		Average weight of mature callus (g)	Average no. of green leaves per culture	Appearance of shoots in culture
	Light	Dark	Light	Dark			
0	70	100	0.717	0.712	0.778	9.57	green
1	80	55	0.544 ns	1.045 ns	-	9.00	green
5	-	-	-	-	-	3.00	green
10	60	95	1.128 ns	0.579 ns	0.772 ns	3.00	green/white
20	65	65	0.128 **	0.432 ns	0.625 ns	1.00	green/white
50	85	50	0.138 **	0.080 ns	0.153 **	1.00	green/white
100	55	70	0.023***	0.030 **	0.055***	0	white
150	70	80	0.021***	0.018 **	-	0	brown/white
200	70	70	-	0.008 **	0.050***	0	brown/white
250	60	65	0.010***	0.010 **	-	0	white
300	80	80	0.006***	0 **	0.036***	0	brown/white
400	-	-	-	-	0.038 **	0	brown/white
500	70	85	0.007***	0.008 **	0.036***	0	brown
600	-	-	-	-	0.034***	-	-
750	-	-	-	-	0.038 **	-	-
1000	-	-	-	-	0.054 **	-	-

*, **, *** = significance at 0.05, 0.01 and 0.001 levels respectively and ns = not significant, as determined by analysis of variance

Appendix III Germination, leaf production and leaf condition on G418[1].

G418 concn (mgl^{-1})	% germination		Average no. of green leaves per plantlet	Average no. of roots per plantlet	Appearance of leaves in culture
	'PLK'	'ELK'			
0	60	80	4.50	1.25	green
5	60	73	3.00 ns	0.25 ns	green
10	53	87	1.00 **	1.25 ns	green, but brown at leaf base and white at leaf tips
20	40	67	0.75 **	0.50 ns	some leaves green, some white, but all vitrified
30	33	87	1.00 **	0 ns	some leaves green, some white, but all vitrified
50	33	67	0.75 **	0 ns	white, leaf bases brown
100	40	67	0.50 ***	0 ns	white or grey-brown

*, **, *** = significance at 0.05, 0.01 and 0.001 probability levels respectively and ns = not significant, as determined by analysis of variance

[1]Unless otherwise stated, the cultivar tested is 'PLK'.

Appendix IV Responses of callusing 'PLK', 'ELK' and 'SWG' seedling stems to G418

G418 concentration (mgl^{-1})	Average weight (± SE) of 'PLK' seedling stem callus[1] (g)		Average weight (± SE) of 'ELK' seedling stem callus (g)		Average weight (± SE) of 'SWG' seedling stem callus (g)	
	Light	Dark[2]	Light	Dark	Light	Dark
0	0.196 ± 0.002	0.121	0.227 ± 0.022	0.297 ± 0.004	0.302 ± 0.030	0.195 ± 0.044
25	0.016 ± 0.003	0.020	0.016 ± 0.007	0.005 ± 0.001	0.014 ± 0.001	0.014 ± 0.001
50	0.009 ± 0.001	0.006	0.010 ± 0.005	0.010 ± 0.003	0.013 ± 0.006	0.007 ± 0.001
75	0.014 ± 0.004	0.012	0.021 ± 0.004	0.009 ± 0.003	0.009 ± 0.004	0.025 ± 0.001
100	0.008 ± 0.003	0.009	0.012 ± 0.005	0.004 ± 0.001	0.006 ± 0.002	0.014 ±0.001
150	0.004 ± 0.003	0.012	0.013 ± 0.001	0.009 ± 0.002	0.016 ± 0.004	0.007 ± 0.002

[1] seedling stems were cultured for six weeks

[2] insufficient samples to calculate standard errors

Appendix V Responses of in vitro-cultured 'PLK' and 'ELK' tissues to hygromycin

Hygromycin concentration (mgl^{-1})	% germination		Average length of longest leaf or hypocotyl (mm)		Average weight of callus produced by seedlings (g)	Average weight of mature 'ELK' callus [1] (g)	Average no. of green leaves per plantlet	Average no. of bleached leaves per plantlet
	'PLK'	'ELK'	'PLK'	'ELK'	'PLK'			
0	75	92	122.8	230	0.289	0.708	2.6	0.20
5	50	83	97.8 ns	162	0.042 ***	0.390	1.2 *	3.00 *
10	42	83	89.4 ns	105.3	0.057 ***	0.402	0 ***	6.40 ***
20	33	100	47.5 *	69.5	0.020 ***	0.154	0 ***	4.20 **
50	33	83	18.8 **	30.4	0 ***	0.100	0 ***	2.80 *
100	42	67	8.9 **	14.1	0 ***	0.087	0 ***	2.00 ns

*, **, *** = significance at the 0.05, 0.01 and 0.001 probability levels respectively and ns = not significant, as determined by analysis of variance

Appendix VI Germination and seedling stem callus production of 'PLK' and 'ELK' on chlorsulfuron

Chlorsulfuron concentration (nM)	% germination		Average weight of 'PLK' seedling stem callus (g)	
	'PLK'	'ELK'	Light	Dark
0	67	90	0.354	0.013
25	60	80	0.013 ***	0.005 **
50	47	80	0.014 ***	0.005 *
75	27	73	0.010 ***	0.004 **
100	47	60	0.008 ***	0.003 **
150	53	73	0.010 ***	0.003 **

*, **, *** = significance at the 0.05, 0.01 and 0.001 levels respectively and ns = not significant as determined by analysis of variance

Appendix VII Responses of *in vitro*-cultured 'PLK', 'ELK' and 'SWG' shoot cultures to chlorsulfuron

Chlorsulfuron concentration (nM)	Average no. of green leaves per plantlet			Average no. of roots per plantlet		
	'PLK'	'ELK'	'SWG'	'PLK'	'ELK'	'SWG'
0	3.25	1.75	3.75	3.25	1.75	3.38
25	0.25 ***	0.25 ***	0.13 **	0.50 ***	1.25 ns	0.25 **
50	0.50 ***	0 ***	0.63 *	1.50 *	0.50 **	0.13 **
75	0 ***	0.50 **	0.13 **	0 ***	0.38 **	0.75 *
100	0 ***	0.75 *	0.50 *	0 ***	0.88 ns	0 **
150	0 ***	0.37 ***	0.13 **	0 ***	0.38 **	0.88 *

*, **, *** = significance at the 0.05, 0.01 and 0.001 levels respectively and ns = not significant as determined by analysis of variance

Appendix VIII Reagents used in Southern Analyses:

Sample buffer:

5 ml glycerol
1 ml 10x TBE (Maniatis *et al.*, 1982)
4 ml H_2O
bromophenol blue (add small amounts until desired intensity of colour is reached)

20x SSC:

175.3 gl^{-1} NaCl (3M final concentration)
88.2 gl^{-1} trisodium citrate (0.3 M final concentration)
Adjust to pH 7.0 with a few drops of 10 N NaOH

1x STE:

10 mM TrisHCl (pH 8.0)
1 mM EDTA (pH 8.0)
100 mM NaCl