PT338

Production of virus resistant potato plants to enable reduced use of insecticides on potatoes

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Know-how for Horticulture™

PT338

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1.0 Introduction

If genetic engineering is to be effective in delivering new potato cultivars, four essential and interacting components are needed; (1) a suitable adventitious shoot (or somatic embryogenic) regeneration system for the cultivars of interest, (2) an efficient transformation system to deliver foreign DNA to individual cells, (3) suitable genes that impart favourable traits and (4) methods to evaluate transgenic plants in the laboratory, glasshouse and the field.

This is the final report for APIC\HRDC project **PT 338** *'Production of virus resistant potato plants to enable reduced use of insecticides on potatoes'* a collaborative project between the Institute for Horticultural Development - Knoxfield (Agriculture Victoria) and the Division of Plant Industry (CSIRO).

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The report is divided into five sections;

- Industry and technical summaries
- Adventitious shoot regeneration
- Transient gene expression
- Production of transgenic plants
- Recommendations

Attached are four appendices, including a list of publications arising from this research.

1.1 Industry summary

Potatoes are a major horticultural crop for which genetic engineering approaches have been successfully used abroad.

A number of genes have been introduced into potato to allow control of a number of important agronomic traits such as virus resistance, susceptibility to bacterial and fungal diseases, insect resistance, the starch content of tubers and susceptibility to tuber bruising.

Potatoes have a number of problems that are difficult or impossible to solve through conventional breeding. Breeding for resistance to potato leaf roll virus (PLRV) is difficult due to sexual incompatibility between the cultivated potato (*Solanum tuberosum*) and known sources of resistance such as *S. brevidens*.

This project had the specific aims of

- developing gene transfer systems for cultivars commonly used as parents in the fresh market component of the National Potato Improvement and Evaluation Scheme (NaPIES),
- transfer genes that could provide resistance to PLRV,
- challenging the resulting plants for their response to PLRV.

Tissue culture systems have been developed to regenerate adventitious shoots for eight cultivars.

Two different gene constructs have been introduced into four cultivars and a population of transgenic plants has been produced.

The transgenic plants are being;

- screened in the laboratory for the presence of the introduced genes,
- grown the glasshouse and challenged with aphids carrying the PLRV.

Importantly, some of the transgenic Sebago lines have greatly reduced levels of virus in inoculated plants and some transgenic lines of 80-90-5 appear to be immune to PLRV.

1.2 Technical summary

A biotechnological approach has been developed to complement conventional breeding for the genetic improvement of potato.

Adventitious shoot regeneration systems have been developed for eight cultivars that are commonly used parents in the fresh market component of the National Potato Improvement and Evaluation Scheme (NaPIES). The important findings are;

- for any set given of conditions, adventitious shoot regeneration from internode explants is better than from leaf explants,
- the optimum regeneration conditions vary between cultivars,
- incorporation of kanamycin at 25 $\mu g\ mL^{-1}$ and above prevented both adventitious shoot regeneration and root growth.

A range of factors that influence gene transfer from *Agrobacterium tumefaciens* to potato have been assessed using transient gene expression assays. The important findings are;

- the optimal cell density of the inoculum,
- the manner in which the explants are inoculated,
- the duration of explant co-cultivation with *Agrobacterium*,
- the presence of acetosyringone during co-cultivation.

Two different gene constructs to control potato leaf roll virus (PLRV) have been prepared. The construct pG10 has the PRLV coat protein driven gene by the CaMV 35S promoter and contains two ribozymes (gene shears) domains targeting the 5' end of the viral genome. The other construct, pRolCpol has the PLRV polymerase driven by a vascular tissue promoter.

A population of transgenic plants has been produced. The cultivars Crystal, Sebago D, 80-90-5 and 80-93-4 have been transformed with pG10. Crystal and 80-90-5 have also been transformed with pRolCpol.

The transgenic plants are being;

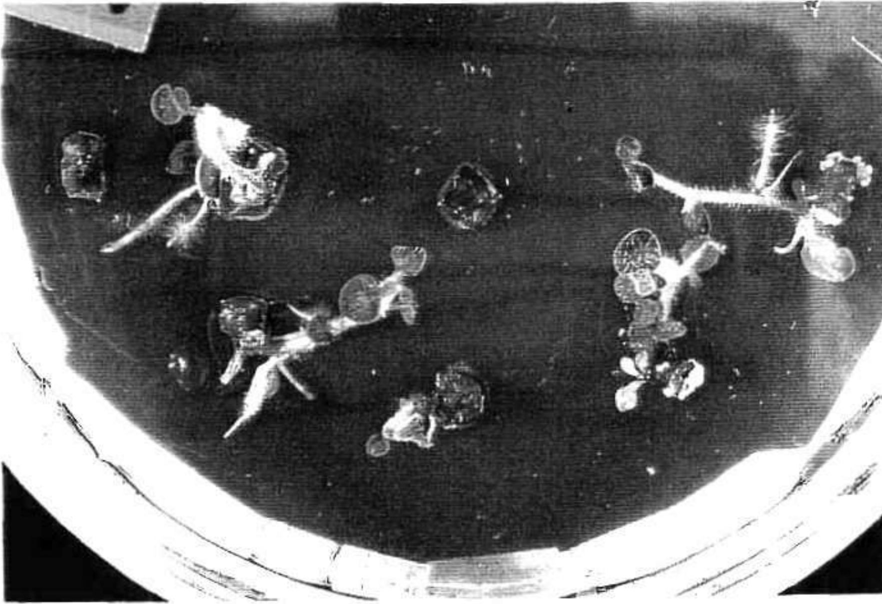
- screened in the laboratory for the presence of the introduced genes,
- grown the glasshouse and challenged with aphids carrying the PLRV.

Some Sebago lines have been produced that have greatly reduced levels of virus in inoculated plants and some lines of 80-90-5 appear to be immune to PLRV.

The areas recommended for further research are;

- confirm the results obtained with Sebago and 80-90-5 with additional glasshouse experiments,
- screen the remaining transgenic plants for response to PLRV infection,
- screen selected lines of Crystal and Sebago D in field trials for agronomic performance with the aim of eventual release to growers,
- determine the sexual transmission of the anti-viral genes through hybridisation to other cultivars and incorporate selected lines into the conventional breeding programme,
- generate additional transgenic lines with new gene constructs for PLRV and PVY in new binary vectors (see 5.3, page 68).

2.0 Adventitious shoot regeneration



Adventitious shoot regeneration from internode explants

2.1 Introduction

The most commonly reported methods for regeneration involve the production of adventitious shoots and/or somatic embryos either directly on an explant (e.g. leaf pieces and internodes) or, alternatively via a callus intermediary. The source of the callus can be cultured explants, protoplasts or cell suspensions. Those shoots or somatic embryos which arise directly from the explant surface are more likely to be genetically identical to the donor tissue (Williams and Maheswaran 1986), whereas those developed via regenerated callus are more prone to somaclonal variation (Larkin and Scowcroft 1981).

With potato, adventitious shoot regeneration has been achieved from a range of explants for a number of cultivars. Most commonly, tuber, leaf, rachis and internode explants have been used which have been derived from *in vitro*, glasshouse or field grown plants (Webb *et al.* 1983; Kikuta and Okazawa 1984; Wheeler *et al.* 1985). Regeneration studies with potato have gone through three distinct phases. Firstly, examples of sporadic and spontaneous shoot regeneration can be found in the literature of the 1950's and 1960's, which in retrospect can be considered scientific curiosities (Lauer and Krantz 1957; Bajaj and Dionne 1967). Secondly, the importance of the cytokinins benzyl amino purine (BAP) and zeatin and gibberellic acid (GA_3) in the regeneration process was determined (e.g. Lam 1977; Jarret *et al.* 1980, 1981). Concomitant research found that regeneration could be improved by initially exposing explants to a callus initiation medium, then transfer to a regeneration medium (Jarret *et al.* 1980; Webb *et al.* 1983). Thirdly, was the finding that plants regenerated from protoplasts resulted in a immense amount of somaclonal variation (Secor and Shepard 1981; Thomas *et al.* 1982). There is no question that potato plants regenerated via a callus intermediary can result in somaclonal variation and it is important to find way to reduce the problem. One reason why regeneration from protoplasts results in somaclonal variation is the time tissue is in a undifferentiated state; the greater the time the greater the incidence of somaclonal variation.

Recently, the importance of ethylene in plant regeneration *in vitro* has been reviewed (Biddington 1992). While there is no clear evidence of its role in potato regeneration it has been reported that the suppression of ethylene production improves protoplast yield and plating efficiency (Perl *et al.* 1988), the growth of shoot cultures (Higgins 1991) and improved transformation (Chang and Chan 1991).

The regeneration systems developed for this project have intentionally been designed to reduce the time explants are exposed to callus initiation. Data presented in this section describe experiments designed to investigate factors affecting adventitious shoot regeneration, including hormonal composition, influence of silver thiosulphate which inhibits ethylene action, type of gelling agent and kanamycin concentration. To a lesser extent factors affecting micropropagation of potato cultivars were investigated. All the cultivars evaluated were commonly used as parental lines in the fresh market component of NaPIES.

2.2 Materials and methods

2.2.1 Cultivar selection

Nine cultivars were nominated at the inaugural meeting of NaPIES (Appendix 1). Five of these (Coliban, Crystal, Sebago, Wilwash and 87-57-9) were available in tissue culture as pathogen tested material and sourced from the Victorian Certified Seed Potato Scheme (now Victorian Seed Potato Authority). The remaining four (66-11-2, 80-90-5, 80-93-4 and 87-12-6) were not in the pathogen tested scheme. It was considered that material used in this project should be of the highest possible health status and should therefore be tested before use.

Tubers of 66-11-2, 80-90-5, 80-93-4 and 87-12-6 were obtained from the National Potato Improvement Centre at IHD-Toolangi. Eye plugs were removed, planted and tested for the following viruses (PVA, PVM, PVS, PVX, PVY and PLRV), potato tuber spindle viroid, the pathogenic bacteria (*Erwinia* spp. [blackleg], *Burkholderia solanacearum* [bacterial wilt] and *Clavibacter michiganense* pv *sepodonicus* [ring rot]) and pathogenic fungi (*Spongospora subterranea* [powdery scab], *Rhizoctonia solani* [black scurf], *Phoma exigua* [gangrene], *Fusarium* spp. [wilt, dry rot], *Verticillium* spp. [wilt] and *Colletotrichum coccodes* [black dot]). Material which tested negative for all these tests was deemed to be of pathogen tested status and introduced into tissue culture using established techniques.

2.2.2 Culture maintenance

Cultures were maintained by subculturing nodes and shoot tips at four week intervals using the salts and organics of Murashige and Skoog (1962) (MS) with 30 gL^{-1} sucrose and 8 gL^{-1} agar (Difco Bacto) supplemented with 8 µM silver thiosulphate (STS) (Chang and Chan 1991). The pH of the medium was adjusted to 5.8 prior to autoclaving at 100 kPa for 20 minutes and STS was added as a membrane filtered solution after autoclaving. Cultures were maintained in 40 x 120 mm polycarbonate tubes at 22 °C with a 16:8 hr (light:dark) photoperiod at a photon flux density of 25 µM m^{-2} sec^{-1}.

2.2.3 Adventitious shoot regeneration

2.2.3.1 General considerations

The regeneration system used comprised two stages, (1) exposure of explants to a callus initiation medium for seven days prior to (2) transfer to regeneration medium. For the series of experiments described the callus initiation medium varied, but the regeneration medium was constant. Therefore all experiments studied the effect of callus initiation media on subsequent regeneration.

All experiments evaluated the regeneration response of internode and leaf explants derived from tissue cultures. Internodes were 1 cm long and leaf explants comprising the distal 1 cm were placed with the abaxial surface in contact with the medium. Experiments were conducted in 9 cm Petri dishes sealed with Parafilm™. Cultures were incubated at 22 °C

with a 16:8 hr (light:dark) photoperiod at a photon flux density of 25 µM m^{-2} sec^{-1} during callus initiation and 75 µM m^{-2} sec^{-1} during regeneration.

A basal medium (BM) comprising MS salts and organics with 30 gL^{-1} sucrose and 8 gL^{-1} agar (Difco Bacto) was used. Supplements used for the callus initiation stage are indicated for the individual experiments. For regeneration, BM was supplemented with 10 µM BAP and 15 µM GA_3 (membrane filtered).

Details of the conditions used are summarised in Appendix 2.

2.2.3.2 Experimental design and analysis

All regeneration experiments comprised five replications with 10 explants per replicate. Each experiment lasted 62 days (seven days on callus initiation media and 55 days on regeneration medium). Observations were made on cultures at frequent intervals; at 7 days an estimate of the percentage callus initiation was made. Qualitative evaluation of regeneration was made at 20 days. Quantitative evaluations were made of shoot regeneration at 31 and 62 days. Data was analysed by analysis of variance and only data from the final evaluation are presented. A regeneration index was established for each cultivar; this is the product of the percentage of explants regenerating adventitious shoots and the mean shoot number of responding explants and represents the number of adventitious shoots that can be regenerated from 100 explants.

2.2.3.3 Effect of cytokinin type and concentration on regeneration of Coliban and Wilwash

The effect of the cytokinins BAP, kinetin, 2iP and TDZ each at 10 µM were tested.

2.2.3.4 Cultivar screening

All selected cultivars were screened using a common approach. The effect of 2 µM BAP and 50 µM BAP with 1 µM α-napthaline acetic acid (NAA) in factorial combination with 4 µM STS was tested.

2.2.3.5 Effect of BAP and NAA on regeneration of 87-57-9

The effect of 2 and 10 µM BAP with and without 0.1 µM NAA was tested.

2.2.3.6 Effect of gelling agent on regeneration of Crystal internode explants

The effect of agar and Gelrite™ (2.5 gL^{-1}) were compared in factorial combination with 2 µM BAP or 50 µM BAP with 1 µM NAA in callus initiation media . The gelling agent was constant for both the callus initiation and regeneration stages.

2.2.3.7 Effect of thiamine concentration and method of sterilisation on regeneration of 80-90-5 internode explants

The effect of membrane filtered thiamine.HCl at 0.3, 3 and 30 μM was tested. For 0.3 μM the control treatment was autoclaved. For callus initiation, 2 μM BAP was used with each experimental medium.

2.2.3.8 Effect of kanamycin concentration on regeneration of Crystal and 80-90-5 internode explants

The effect of kanamycin at 0, 25, 50 and 100 μg mL^{-1} during regeneration was tested. For callus initiation BM was supplemented with 2 μM BAP and 4 μM STS and no kanamycin was used.

2.2.4 Micropropagation experiments

2.2.4.1 Effect of STS and gelling agent on growth of 87-12-6

Nodal explants were transferred to BM with and without 8 μM STS in factorial combination with agar or Gelrite™. Each of the four treatments was replicated 15 times.

2.2.4.2 Effect of kanamycin concentration on shoot and root growth

The effect of supplementing BM with 0, 25, 50 and 100 μg mL^{-1} kanamycin was tested with the cvs. Crystal, Sebago, 80-93-4 and 80-90-5.

2.3 Results

2.3.1 Adventitious shoot regeneration

2.3.1.1 Effect of cytokinin type and concentration on regeneration of Coliban and Wilwash

Callus was initiated from internode and leaf explants of both cultivars at 90 to 100% frequency with BAP, kinetin, 2iP and TDZ. On transfer to regeneration medium, only internode explants initially cultured with 10 μM BAP regenerated shoots at a frequency of less than 10%.

While none of the media tested achieved a high percentage of regeneration, the results indicate that regeneration could be obtained from both cultivars, that internode explants was superior to leaf explants and that BAP is a suitable cytokinin source for callus initiation.

2.3.1.2 Cultivar screening

Results from cultivar screening are presented in Table 1a to 1h. In all cases the number of shoots regenerated was greater from internode than leaf explants and only data from the former is presented.

2.3.1.2.1 Coliban

Shoot regeneration was greater from internodes exposed to 2 µM BAP with 4 µM STS (92%) than 2 µM BAP alone (72%) and both of these treatments were significantly better than the treatments with 50 µM BAP, 1 µM NAA ± 4 µM STS (Table 1a). While the mean number of regenerated shoots is virtually identical with the 2 µM BAP ± 4 µM STS treatments (*ca.* 3.4) a callus initiation medium with STS results a higher percentage of regenerating explants.

2.3.1.2.2 Crystal

All callus initiation media tested resulted in a high percentage of shoot regeneration with no significant difference between them. When shoot number was evaluated, media with 2 µM BAP ± 4 µM STS were significantly better than media containing 50 µM BAP, 1 µM NAA ± 4 µM STS (Table 1b).

2.3.1.2.3 Sebago

As with Crystal, internode explants of Sebago regenerated adventitious shoots at a high frequency with all callus initiation media tested. While callus initiation media with 50 µM BAP, 1 µM NAA ± 4 µM STS resulted in 100% shoot regeneration, it is the medium with 2 µM BAP that is superior due to the greater number of regenerated shoots (Table 1c).

2.3.1.2.4 Wilwash

Adventitious shoot regeneration was significantly better from internode explants exposed to 2 µM BAP with 70% of explants responding producing a mean shoot number of 2.6 (Table 1d).

2.3.1.2.5 80-90-5

As with Crystal all callus initiation media tested resulted in a high percentage of shoot regeneration with no significant difference between them. When shoot number was evaluated, medium with 2 µM BAP plus 4 µM STS was significantly better than the other media tested (Table 1e).

2.3.1.2.6 80-93-4

The callus initiation medium with 2 μM BAP was significantly better than the other three tested with 90% of the explants responding producing a mean shoot number of 9.9 (Table 1f).

2.3.1.2.7 87-12-6

Shoot regeneration was high and significantly better from three of the four callus initiation media tested (2 μM BAP ± 4 μM STS and 50 μM BAP, 1 μM NAA). The preferred callus initiation media are 2 μM BAP ± 4 μM STS due to the number of shoots regenerated (Table 1g).

2.3.1.2.8 87-57-9

Shoot regeneration was extremely low (5%) and only obtained with 2 μM BAP (Table 1h).

2.3.1.3 Effect of BAP and NAA on regeneration of 87-57-9

Due to the poor regeneration obtained with 87-57-9 during the cultivar screening experiments (see Table 1h) it was decided to test additional callus initiation media. The effects of 2 μM BAP ± 0.1 μM NAA and 10 μM BAP ± 0.1 μM NAA were evaluated. Compared to the best callus initiation medium from the previous experiment (2 μM BAP) it was possible to increase shoot regeneration to 32% with a mean shoot number of 2.1 shoots by using 10 μM BAP with 0.1 μM NAA (Table 2). While the level of regeneration is a substantial improvement to that reported previously, it is still low compared to some of the results obtained for other cultivars.

2.3.1.4 Summary of adventitious regeneration experiments

From the cultivar screening experiments (sections 2.3.1.2.1 to .8 and 2.3.1.3) it is possible to determine a preferred callus initiation medium and a regeneration index for each cultivar.

The eight cultivars all have a preferred callus initiation media based on 2 μM BAP ± STS, with the exception of 87-57-9, where the best callus initiation medium tested is 10 μM BAP with 0.1 μM NAA (Table 1i).

Using a regeneration index it is possible to rank each cultivar. The cultivars 80-93-4, Sebago, 80-90-5, 87-12-6 and Crystal can be considered highly regenerable, the cultivars Coliban and Wilwash are moderately regenerable and 87-57-9 is poorly regenerable (Table 1j).

2.3.1.5 Effect of gelling agent on regeneration of Crystal internode explants

The importance of altering the gelling agent was related to the callus initiation medium used. With 2 µM BAP the gelling agent used had no effect on the percentage of explants which regenerated, but the number of shoots produced was significantly better with agar. When 50 µM BAP, 1 µM NAA was used for callus initiation, shoot regeneration and shoot number were significantly better with agar (Table 3). The disparity in shoot number with 50 µM BAP, 1 µM NAA with agar in Tables 1b and 3 can be accounted for. With the experiment described in Table 3, two of the five replicates were lost due to contamination and one of the remaining replicates had a extraordinarily high level of regenèration. This is of no major consequence as the preferred callus initiation medium is 2 µM BAP + STS.

2.3.1.6 Effect of thiamine concentration and method of sterilisation on regeneration of 80-90-5 internode explants

There was no significant difference of thiamine.HCl concentration or its mode of sterilisation on shoot regeneration (Table 4).

2.3.1.7 Effect of kanamycin concentration on regeneration of Crystal and 80-90-5 internode explants

For the two cultivars tested the incorporation of kanamycin into the regeneration medium at 25 µg mL^{-1} and above prevented any regeneration of adventitious shoots. After 20 days of culture (seven on callus initiation [without kanamycin] and 13 on regeneration [with kanamycin]) it was found that callus initiation decreased as kanamycin concentration increased (Table 5).

2.3.2 Micropropagation experiments

2.3.2.1 Effect of STS and gelling agent on growth of 87-12-6

Line 87-12-6 did not grow as vigorously as the other eight nominated cultivars when grown on maintenance medium (BM plus STS). Growth (as determined by stem length and leaf number) was significantly improved by omitting STS, irrespective of gelling agent (Table 6). The preferred maintenance medium for 87-12-6 is MS salts and organics with 30 gL^{-1} sucrose and 2.5 gL^{-1} Gelrite™.

2.3.2.2 Effect of kanamycin concentration on shoot and root growth

The addition of kanamycin completely prevented root growth for the four cultivars tested, whereas omitting kanamycin resulted in 100% rooting (data not presented). The number and mean length of roots varied between cultivar; Crystal and 80-93-4 produced more and longer roots than Sebago and 80-90-5 (Table 7). With each of the four cultivars, shoot length and leaf number decreased with increasing kanamycin concentration (Table 7).

2.4 Discussion

Each of the eight potato cultivars tested was capable of regenerating adventitious shoots, the efficiency of which was dependent on the hormonal composition of the callus initiation medium and the cultivar used.

A comparison of cytokinin types established that callus induced in the presence of the cytokinin BAP was superior to others tested for shoot regeneration. Zeatin has been successfully used as a cytokinin source by others (Jarret *et al* 1980; Kikuta and Okazawa 1984) and that it was unsuccessful in our experiments may be due to different cultivars and\or explants being used.

The two major conclusions from the cultivar screening experiments were the differing cultivar responses to a standard set of conditions (Table 1a-h) and the extremely low rate of regeneration from leaf explants. Indeed, regeneration from leaf explants was so poor the data has not been presented or analysed.

Regeneration studies with a range of plant species, including potato have found differences in the way that cultivars respond (Webb *et al.* 1983; Wheeler *et al.* 1985). The exact reasons why this variation occurs are unknown, but in some species, for example, wheat, tomato and *Solanum phureja* it has a genetic component (Lazar *et al.* 1984; Koornneef *et al.* 1987; Taylor and Veilleux 1992) and this is most the likely reason for the variation observed.

A range of explants have been used successfully to regenerate potatoes, including leaf, rachi, tuber and internodes (Webb *et al.* 1983; Kikuta and Okazawa 1984; Wheeler *et al.* 1985). Why leaf explants are not suitable with the nominated cultivars could be genetic or more likely related to the regeneration system used. Previous research comparing different explant sources with, for example, carnation (Miller *et al.* 1991), chrysanthemum (Kaul *et al.* 1990) and *Brassica napus* (Julliard *et al.* 1992) cultivars found that different explant types vary in their ability to regenerate. The differences in the ability of leaf and internode explants to regenerate could reflect different endogenous hormone levels already present in the explants which might influence responsiveness to exogenously applied growth regulators (Hussey 1978; Carnes *et al.* 1988).

Plants in tissue culture produce ethylene which can accumulate in the culture container (Gamborg and La Rue 1968) and have a stimulatory or inhibitory affect on growth depending on species. Incorporation of $AgNO_3$ improved shoot regeneration from wheat and *Nicotiana plumbaginifolia* callus cultures due to a inhibition of ethylene action (Purnhauser *et al.* 1987). The precise role of ethylene and STS in these experiments is related to cultivar and the hormonal composition of the callus initiation medium. The cultivars Wilwash and 80-93-4 are possibly ethylene responsive as STS incorporated in the callus initiation medium reduced shoot regeneration. A similar observation has previously been made (Hulme *et al.* 1992) where regeneration was only possible or improved by using STS.

Regeneration of 87-57-9 could be improved, *albeit* slightly, by altering the concentration of the growth regulators for callus initiation. Manipulation of the growth regulator component of media is frequently used to alter regeneration response and has been successfully used with numerous species (George 1993). That regeneration was only slightly improved is an indication that other components of the callus initiation media need to be tested if the regeneration level of 87-57-9 is to increased.

For many years agar has been the gelling agent of choice for plant tissue culture. There are some reports of other gelling agents, in particular Gelrite™, being superior for growth and regeneration (e.g. Ichi *et al.* 1986; de Wald *et al.* 1989) which has been associated with improved nutrient uptake from the medium (Almehdi and Parfitt 1986). For the cultivar Crystal, agar was superior to Gelrite™ with both callus initiation media tested, which could reflect that nutrient availability is not a problem with agar gelled media or that Gelrite™ may be inhibitory to regeneration. The effect of gelling agent was also evaluated for the routine micropropagation of 87-12-6. Growth was significantly improved with Gelrite™ and this could be associated with improved nutrient uptake. A comparison of the different responses between the gelling agents in the regeneration and micropropagation experiments is not valid as the tissue culture system and mode of nutrient uptake are different.

Incorporation of kanamycin at 25 $\mu g\ mL^{-1}$ and above prevented both adventitious shoot regeneration and root growth. Kanamycin is a commonly used selectable marker for plant transformation (Angenon *et al.* 1994) and it is important to establish the sensitivity and response of potato prior to commencing detailed transformation studies. Plants differ in their sensitivity to antibiotics, for example in grapevine cultures, 7 $\mu g\ mL^{-1}$ kanamycin completely inhibited callus formation, root 'initiation' and adventitious shoot formation (Colby and Meredith 1990) whereas with sugarbeet 150 $\mu g\ mL^{-1}$ kanamycin was required to inhibit callus growth (Catlin 1990). The data obtained suggest that a concentration of 25 $\mu g\ mL^{-1}$ would be sufficient for the selection of transformed cells.

These results clearly demonstrate that by using a standardised and systematic approach successful adventitious shoot regeneration can be readily achieved from potato cultivars. Factors such as cultivar, the callus initiation medium and the explant have a major influence on regeneration, whereas others, such as gelling agent have only a minor influence.

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Table 1a. Effect of callus initiation media on shoot regeneration from internodes of Coliban

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	72	2.5	3.4
2 μM BAP + STS	92	3.3	3.5
50 μM BAP, 1 μM NAA	16	0.4	2.6
50 μM BAP, 1 μM NAA + STS	4	0.1	0.3

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Main effects

Medium	Shoot regeneration (%)	Shoot number	
		Mean of total	Mean of responding
2 μM BAP	82	2.9	3.5
50 μM BAP, 1 μM NAA	10	0.3	1.5
No STS	44	1.5	3.0
+ STS	48	1.7	1.9

Table 1b. Effect of callus initiation media on shoot regeneration from internodes of Crystal

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	90	4.8	5.4
2 μM BAP + STS	100	5.5	5.5
50 μM BAP, 1 μM NAA	82	2.5	2.9
50 μM BAP, 1 μM NAA + STS	96	3.2	3.3

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Main effects

Medium	Shoot regeneration (%)	Shoot number	
		Mean of total	Mean of responding
2 μM BAP	95	5.2	5.5
50 μM BAP, 1 μM NAA	89	2.9	3.1
No STS	86	3.7	4.2
+ STS	98	4.4	4.4

Table 1c. Effect of callus initiation media on shoot regeneration from internodes of **Sebago**

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	92	7.5	8.4
2 μM BAP + STS	82	5.5	6.9
50 μM BAP, 1 μM NAA	100	5.1	5.1
50 μM BAP, 1 μM NAA + STS	100	3.8	3.8

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Main effects

Medium	Shoot regeneration (%)	Shoot number	
		Mean of total	Mean of responding
2 μM BAP	87	6.5	7.7
50 μM BAP, 1 μM NAA	100	4.5	4.5
No STS	96	6.3	6.8
+ STS	91	4.6	5.4

Table 1d. Effect of callus initiation media on shoot regeneration from internodes of **Wilwash**

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	70	1.8	2.6
2 μM BAP + STS	34	0.5	1.3
50 μM BAP, 1 μM NAA	22	0.3	1.3
50 μM BAP, 1 μM NAA + STS	8	0.1	0.8

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Main effects

Medium	Shoot regeneration (%)	Shoot number	
		Mean of total	Mean of responding
2 μM BAP	52	1.2	2.0
50 μM BAP, 1 μM NAA	15	0.2	1.1
No STS	46	1.1	2.0
+ STS	21	0.3	1.1

Table 1e. Effect of callus initiation media on shoot regeneration from internodes of **80-90-5**

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 µM BAP	96	4.7	4.9
2 µM BAP + STS	100	6.9	6.9
50 µM BAP, 1 µM NAA	100	4.8	4.8
50 µM BAP, 1 µM NAA + STS	98	3.8	3.9

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Main effects

Medium	Shoot regeneration (%)	Shoot number	
		Mean of total	Mean of responding
2 µM BAP	98	5.8	5.9
50 µM BAP, 1 µM NAA	99	4.3	4.4
No STS	98	4.8	4.9
+ STS	99	5.4	5.4

Table 1f. Effect of callus initiation media on shoot regeneration from internodes of **80-93-4**

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	90	9.2	9.9
2 μM BAP + STS	58	3.1	5.6
50 μM BAP, 1 μM NAA	70	2.0	2.9
50 μM BAP, 1 μM NAA + STS	40	1.0	2.5

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Main effects

Medium	Shoot regeneration (%)	Shoot number	
		Mean of total	Mean of responding
2 μM BAP	74	6.2	7.8
50 μM BAP, 1 μM NAA	55	1.5	2.7
No STS	80	5.6	6.4
+ STS	49	2.1	4.1

Table 1g. Effect of callus initiation media on shoot regeneration from internodes of 87-12-6

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	84	5.7	6.9
2 μM BAP + STS	100	5.4	5.4
50 μM BAP, 1 μM NAA	8	0.08	0.4
50 μM BAP, 1 μM NAA + STS	90	2.6	2.9

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Main effects

Medium	Shoot regeneration (%)	Shoot number	
		Mean of total	Mean of responding
2 μM BAP	92	5.6	6.2
50 μM BAP, 1 μM NAA	49	1.3	1.7
No STS	46	2.9	3.7
+ STS	95	4.0	4.2

Table 1h. Effect of callus initiation media on shoot regeneration from internodes of **87-57-9**

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	5	0.1	0.3
2 μM BAP + STS	0	0	0
50 μM BAP, 1 μM NAA	0	0	0
50 μM BAP, 1 μM NAA + STS	0	0	0

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Table 1i. Preferred callus initiation medium for each cultivar [1]

Cultivar	Medium
Coliban	2 µM BAP + STS
Crystal	2 µM BAP + STS
Sebago	2 µM BAP
Wilwash	2 µM BAP
80-90-5	2 µM BAP + STS
80-93-4	2 µM BAP
87-12-6	2 µM BAP ± STS[2]
87-57-9	10 µM BAP + 0.1 µM NAA

[1] Based on the regeneration index

[2] Due to similarity of the regeneration index

Table 1j. Regeneration index for nominated cultivars

Category [1]	Cultivar	Regeneration index [2]
Highly regenerable	80-93-4	891
	Sebago	773
	80-90-5	690
	87-12-6	580
	Crystal	550
Moderately regenerable	Coliban	322
	Wilwash	182
Poorly regenerable	87-57-9	67

[1] Arbitaraly based on a regeneration index of 400+ being highly responsive, 100 to 399 being moderately responsive and < 99 being poorly responsive.

[2] Product of shoot regeneration (%) and mean shoot number of responding explants for preferred callus initiation medium for each cultivar. The regeneration index represents the number of adventitious shoots that can be regenerated from 100 explants.

Table 2. Effect of callus initiation media on shoot regeneration from internodes of 87-57-9

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 μM BAP	4	0.1	0.6
2 μM BAP + 0.1 NAA	12	0.3	1.8
10 μM BAP	18	0.3	1.0
10 μM BAP + 0.1 μM NAA	32	0.7	2.1

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Table 3. Effect of gelling agent and callus initiation media on shoot regeneration from internodes of Crystal

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
2 µM BAP + Agar	74	3.7	4.9
2 µM BAP + Gelrite	74	2.3	3.0
50 µM BAP, 1 µM NAA + Agar	97	6.2	6.5
50 µM BAP, 1 µM NAA + Gelrite	70	2.3	3.2

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

Table 4. Effect of thiamine.HCl concentration and method of sterilisation on shoot regeneration from internodes of 80-90-5

Medium	Shoot regeneration [1] (%)	Shoot number	
		Mean of total [2]	Mean of responding [3]
0.3 µM thiamine, A [4]	100	7.0	7.0
0.3 µM thiamine, M	100	8.8	8.8
3.0 µM thiamine, M	100	7.8	7.8
30 µM thiamine, M	100	9.3	9.3

[1] Percentage of explants regenerating adventitious shoots.

[2] Mean shoot number for all explants.

[3] Mean shoot number for responding explants.

[4] A = autoclaved, M= membrane filtered.

Table 5. Effect of kanamycin concentration on shoot regeneration from internodes of Crystal and 80-90-5

Cultivar: Crystal

Kanamycin concentration ($\mu g\ mL^{-1}$)	Callus initiation [1] (%)	Shoot regeneration [2] (%)	Shoot number	
			Mean of total [3]	Mean of responding [4]
0	100	93	4.0	4.3
25	86	0	0	0
50	60	0	0	0
100	45	0	0	0

Cultivar: 80-90-5

Kanamycin concentration ($\mu g\ mL^{-1}$)	Callus initiation [1] (%)	Shoot regeneration [2] (%)	Shoot number	
			Mean of total [3]	Mean of responding [4]
0	97	70	4.9	6.9
25	78	0	0	0
50	56	0	0	0
100	46	0	0	0

[1] Percentage of explants initiating callus after 20 days.

[2] Percentage of explants regenerating adventitious shoots.

[3] Mean shoot number for all explants.

[4] Mean shoot number for responding explants.

Table 6. Effect of gelling agent and STS on shoot growth of 87-12-6

Treatment	Stem length (cm)	Leaf number
Agar + STS	3.7	4.7
Agar	7.2	11.7
Gelrite + STS	7.8	11.1
Gelrite	9.8	10.6

n = 15

Main effects

Treatment	Stem length (cm)	Leaf number
Agar	5.5	8.2
Gelrite	8.8	10.9
No STS	8.5	11.2
+ STS	5.6	7.9

Table 7. Effect of kanamycin concentration on root and shoot growth of four potato cultivars

Cultivar: Crystal

Kanamycin concentration (µg\mL)	Root number	Root length		Shoot length (cm)	Leaf number
		Total (cm)	Mean (cm)		
0	5	70	6.2	6.2	9
25	0	0	0	4.2	8
50	0	0	0	2.8	7
100	0	0	0	2.4	6

Cultivar: Sebago

Kanamycin concentration (µg\mL)	Root number	Root length		Shoot length (cm)	Leaf number
		Total (cm)	Mean (cm)		
0	2	38	17.2	5.1	8
25	0	0	0	2.3	4
50	0	0	0	2.6	4
100	0	0	0	2.2	5

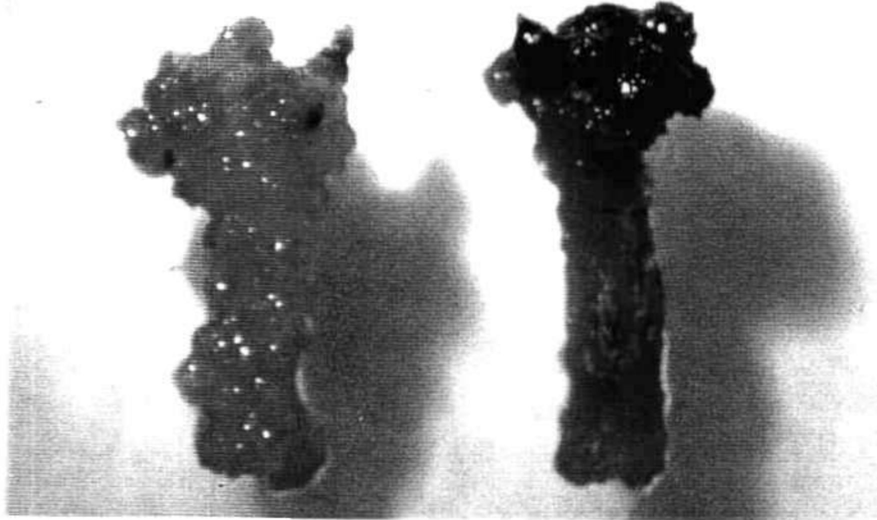
Cultivar: 80-90-5

Kanamycin concentration (µg\mL)	Root number	Root length		Shoot length (cm)	Leaf number
		Total (cm)	Mean (cm)		
0	2	32	5.4	5.4	5
25	0	0	0	3.3	5
50	0	0	0	2.9	4
100	0	0	0	2.7	5

Cultivar: 80-93-4

Kanamycin concentration (µg\mL)	Root number	Root length		Shoot length (cm)	Leaf number
		Total (cm)	Mean (cm)		
0	8	87	11.7	6.8	7
25	0	0	0	3.9	5
50	0	0	0	2.6	4
100	0	0	0	3.2	5

3.0 Transient gene expression



gus gene expression in internodes after co-cultivation with p35S GUSintron

3.1 Introduction

The production of transgenic plants is a time consuming and complex process. When using *Agrobacterium* as a gene transfer system a cascade of events occur. Briefly, phenolic compounds produced by wounded plant cells initiate the transfer of T-DNA from the *Agrobacterium* through the induction of the bacterial *vir* genes. The T-DNA, as a single stranded molecule, moves through the bacterial membrane and is transferred to the plant cell where it moves to the nucleus and is integrated at random (Zambryski 1992). Once this has occurred it is then necessary for the transgenic cells to regenerate to shoots thence plants. From the original inoculation of bacterial and plant cells to the eventual development and growth of a transgenic plant can take about four months, depending on species.

From a practical point, the transfer of genes, when using *Agrobacterium*, involves two distinct stages, (1) the manner in which the bacterial cells are inoculated with a explant and (2) the duration of the co-cultivation period. Because of the complexity of the process it has been difficult to study. With the development of the GUS reporter system (Jefferson 1987) it is possible to more easily study factors that influence gene transfer as the gene product can be readily detected with a simple histochemical assay.

Data presented in this section result from a series of experiments designed to evaluate factors that could influence gene transfer. The *gus* gene used has been modified to minimise gene expression in *Agrobacterium*. These modifications include alteration of the Shine-Dalgarno ribosome binding site preceding the *gus* gene, so that mRNA could not be efficiently translated (Jansen and Gardner 1989) or to a portable plant intron in the *gus* gene (Vancanneyt *et al.* 1990) so that the expression seen can be attributed to the treatment imposed.

3.2 Materials and methods

3.2.1 General considerations

Experiments were conducted with the cultivars Coliban, Sebago and Wilwash using internode and leaf explants as indicated and prepared as described in section 2.2.3.1.

3.2.2 Gene constructs and growth of *Agrobacterium*

The gene constructs p35S GUSintron (Vancanneyt *et al.* 1990) and pKIWI 105 (Jansen and Gardner 1989) were used. They were transferred to the *Agrobacterium* strain LBA 4404 (Hoekema *et al.* 1985) using *ménage à trois* with the helper strain pRK 2013 (Ditta *et al.* 1980).

Cultures were grown on LB medium with appropriate antibiotics on 9 cm Petri plates for 48 hrs at 26 °C. Cells were flushed off the Petri plates with 20 mL of Murashige and Skoog (1962) medium with 30 gL^{-1} sucrose (MS) and used for experiments. Explants were co-cultivated for two days on solidified MS medium prior to histochemical staining.

3.2.3 Histochemical staining and evaluation of experiments

Explants were transferred to 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) in 100 mM phosphate buffer (pH 7), incubated at 37 °C for 24 hr and stored in 70% ethanol. The number of blue spots was counted using a binocular microscope.

3.2.4 Effect of inoculation method

Internode explants were added to *Agrobacterium* cells and either kept under a gentle vacuum for 1 min, gently agitated or kept static for 5 min. They were blotted dry and transferred to solidified MS medium for two days prior to histochemical staining.

3.2.5 Effect of co-cultivation time

Internode explants were prepared and inoculated using a gentle vacuum (see above), blotted dry and co-cultivated for two or three days on solidified MS medium prior to histochemical staining.

3.2.6 Effect of acetosyringone

For this experiment *Agrobacterium* were grown by transferring a loop of culture to 20 mL LB medium with appropriate antibiotics and growing overnight at 28 °C with shaking. Acetosyringone (100 µM) was added to the *Agrobacterium* growth medium and\or the co-cultivation medium. Internode explants were transferred to the bacterial solution for five minutes, blotted dry and transferred to appropriate co-cultivation media for two days prior to histochemical staining.

3.2.7 Effect of cell density

Bacterial cells were prepared by flushing them from a Petri plate, after which they were centrifuged and resuspended in MS medium to various cell densities. Leaf and internode explants were prepared in the *Agrobacterium* cells, blotted dry, co-cultivated for three days on MS medium then transferred to callus initiation medium for an additional seven days, at which stage the experiment was terminated.

3.2.8 Effect of time after inoculation

Bacterial cells were prepared as above and diluted to a cell density of 2.10^5 cells mL^{-1}. Leaf and internode explants were processed as above except that explants were assessed after 1, 2, 3, 4, 6, 8 or 10 days. At each day after inoculation 50 explants of each type were harvested.

3.3 Results

3.3.1 Effect of inoculation method

The manner in which internode explants were inoculated with *Agrobacterium* influenced gene transfer. Explants kept under a gentle vacuum for one minute resulted in a greater number of transformation events, with both gene constructs, than if kept static or gently agitated (Table 8)

3.3.2 Effect of co-cultivation time

Internode explants of Coliban and Wilwash co-cultivated for three days resulted in a greater number of transformation events (Table 9).

3.3.3 Effect of acetosyringone

The presence of acetosyringone during various stages of co-cultivation influences gene transfer. Incorporating 100 μM acetosyringone into the co-cultivation medium resulted in the maximum number of transformation events with both gene constructs (Table 10).

3.3.4 Effect of cell density

The two most important findings were that a minimum number of bacterial cells are required before gene transfer occurs (or at least is observable) and there are two orders of magnitude difference in the way internode and leaf explants respond (Table 11). With internode explants a density of $2. 10^5$ cells per mL achieved the most transformation events whereas with leaf explants a cell density of $2. 10^7$ was required.

3.3.5 Effect of time after inoculation

Using an inoculum with a cell density of $2. 10^5$ cells per mL the maximum number of transformation events with internode explants occured between four and six days after inoculation and with leaf explants between four and eight days (Table 12). With internodes there was no evidence of gene transfer two days after inoculation, with the number of transformation events increasing until six days after which it declines. A similar trend occured with leaf explants. This result is in apparent conflict with the data presented in Table 9 where there was evidence of gene transfer after two days co-cultivation.

3.4 Discussion

A variety of factors have been found to influence gene transfer from *Agrobacterium* to potato explants. The most common approach to inoculate explants is to prepare them in advance and then add the inoculum and leave for a designated period, ranging from minutes to hours before transfer to co-cultivation medium (Mathis and Hinchee 1994). This method resulted in the least number of transformation events. Imposing a gentle vacuum for 1 min was the best treatment due to improved contact between the *Agrobacterium* and the

explant. Such a method is technically demanding as it is difficult to maintain sterility. A viable alternative is to submerge the plant tissue in the *Agrobacterium* solution and prepare the explants as normal. This approach takes advantage of a pressure difference between the inside and outside of plant tissue, so that when it is cut under a solution only the solution enters the plant.

The correct co-cultivation time is a matter of compromise. It can be argued the longer the time the greater the number of transformation events, but the major disadvantage is excessive growth of *Agrobacterium* cells making their subsequent removal difficult. A co-cultivation time of two to three days has been used successfully with a number of plant species including; *Medicago varia* (alfalfa) (Chabaud *et al.* 1988), *M. truncatula* (barrel medic) (Chabaud *et al.* 1996), *Glycine max* (soybean) (Hinchee *et al.* 1988) and *Vigna mungo* (Karthikeyan *et al.* 1996). With the cultivars Coliban and Wilwash there is evidence of gene transfer after two days co-cultivation yet with Sebago the first evidence of transformation is found after three days. This difference could be a function of cultivar differences or more likely that the experiments with Sebago used a known and partially optimised cell density.

Addition of 100 µM acetosyringone to the co-cultivation medium increased the number of transformation events due to the induction of *vir* genes in *Agrobacterium* (Stachel *et al.* 1985). There are varying reports on the value of acetosyringone for improving gene transfer. For some species, such as *Antirrhinum majus* (snapdragon), the usefulness of acetosyringone was related to the pH of the co-cultivation medium (Holford *et al.* 1992), whereas with *G. max* it was less dependent on medium pH and a function of the *Agrobacterium* strain used (Godwin *et al.* 1991). With apple there was only a benefit if acetosyringone was used in conjunction with a osmoprotectant during the growth of the *Agrobacterium* cells (James *et al.* 1993). These results clearly show the complexity of *vir* gene induction and the importance of transient expression systems to study them.

Cell density is a frequently overlooked but important factor affecting gene transfer. Excess bacterial cells can result in plant cells becoming stressed resulting in reduced gene uptake and too few cells can result in limited transgenic shoot production due to less cells being transformed (Curtis *et al.* 1995). The effect of cell density on transformation has not been extensively studied but is important with tomato (Fillatti *et al.* 1987) and critical with lettuce (Michelmore *et al.* 1987).

Collectively, the information generated from these transient expression studies provides a set of conditions, that could be suitable for gene transfer from *Agrobacterium* to potato. Suitable conditions include preparing *Agobacterium* at a cell density 2. 10^5 cell mL^{-1}, preparing explants in the *Agrobacterium* solution then transferring to a co-cultivation containing 100 µM acetosyringone for three days.

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Table 8. Effect of inoculation method on the number of transformation events using the cultivar Wilwash

Inoculation method	Gene construct	
	p35S GUSintron	pKIWI 105
Vacuum	3.0	2.4
Agitated	1.6	2.3
Static	0.1	0.4

n = 50

Table 9. Effect of co-cultivation time on the number of transformation events using p35S GUSintron

Cultivar	Co-cultivation time (days)	
	Two	Three
Coliban	2.3	3.4
Wilwash	2.7	3.9

n = 50

Table 10. Effect of acetosyringone in the *Agrobacterium* growth and\or co-cultivationmedium on the number of transformation events using the cultivar Wilwash

Acetosyringone present		Gene construct	
***Agrobacterium* growth medium**	**Co-cultivation medium**	**p35S GUSintron**	**pKIWI 105**
Yes	No	0.6	0.4
No	Yes	3.5	2.3
Yes	Yes	1.7	1.9
No	No	0	0.5

n = 50

Table 11. Effect of *Agrobacterium* cell density on the number of transformation events using internode and leaf explants with the cultivar Sebago

Internode	Cell density (cells mL^{-1})				
	$2.\ 10^9$	$2.\ 10^7$	$2.\ 10^5$	$2.\ 10^3$	20
Mean no. blue spots per cut end	1.1	1.8	2.9	0	0
% explants with blue spots	69	75	90	0	0
Mean no. blue spots per responding end	1.7	2.4	3.2	0	0
% explants initiating callus	63	85	96	0	0

Leaf	Cell density (cells mL^{-1})				
	$2.\ 10^9$	$2.\ 10^7$	$2.\ 10^5$	$2.\ 10^3$	20
Mean no. blue spots per explant	1.5	2.0	0.5	0	0
Mean no. blue spots per responding explant	1.9	2.2	1.9	0	0
% explants with blue spots	80	90	26	0	0
% explants initiating callus	0	0	0	0	0

After histochemical staining the number of blue spots and explants with callus were determined. For internode explants the results have been presented on the basis of a 'cut end' or responding end as adventitious shoot regeneration occurs only from one end of the explant. This type of measurement is therefore a more realistic indication of what may occur during the regeneration of transgenic plants. n = 50 for each treatment and explant type.

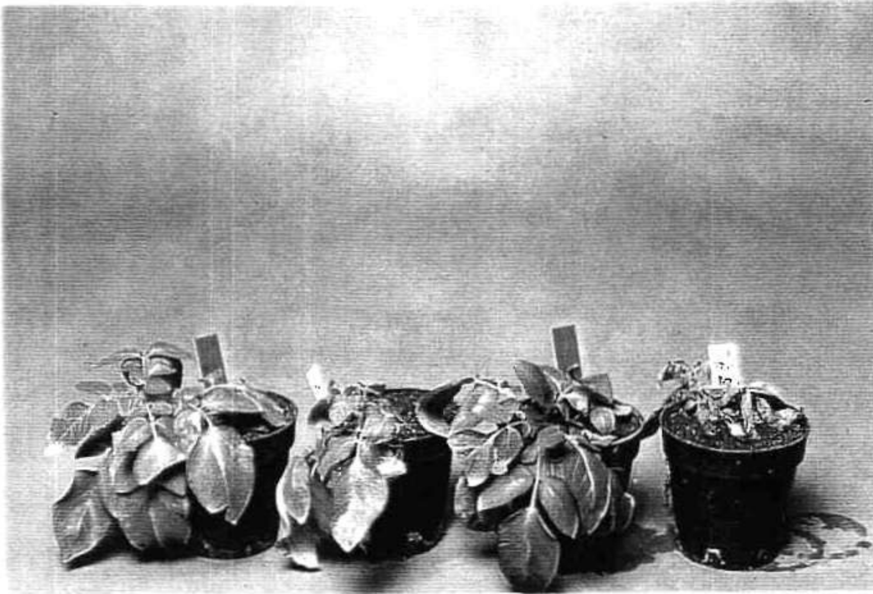
Table 12. Effect of time on the number of transformation events using internode and leaf explants with the cultivar Sebago

Internode	Days after inoculation						
	1	2	3	4	6	8	10
Mean no. blue spots per cut end	0	0	4.7	10.6	11.4	9.5	2.3
% explants with blue spots	0	0	80	99	100	99	52.3
Mean no. blue spots per responding explant	0	0	5.9	10.7	11.4	9.6	3.5

Leaf	Days after inoculation						
	1	2	3	4	6	8	10
Mean no. blue spots per explant	0	0	1.2	1.6	1.5	2.0	0.6
Mean no. blue spots per responding explant	0	0	3.7	3.0	2.0	2.9	1.6
% explants with blue spots	0	0	32	55	72	67	37

After histochemical staining the number of blue spots and explants with callus were determined. For internode explants the results have been presented on the basis of a 'cut end' or responding end as adventitious shoot regeneration occurs only from one end of the explant. This type of measurement is therefore a more realistic indication of what may occur during the regeneration of transgenic plants. n = 50 for each treatment and explant type.

4.0 Production of transgenic plants



Transgenic potato plants

The plants on the left side and second from right are transgenic and show immunity to virus infection. The other two are non-transgenic and are susceptible

4.1 Introduction

The first transgenic plants derived from *Agrobacterium*-mediated gene transfer were obtained simultaneously and independently using protoplasts (de Block *et al.* 1984; Horsch *et al.* 1984). However, it was the development of the 'leaf disc' system (Horsch *et al.* 1985) which has revolutionised the rapid developments in transformation over the last 12 years. This is largely because it is technically easier to regenerate adventitious shoots (thence whole plants) from explants, such as leaves and internodes, than from protoplasts. Because of the economic importance of potato (Coombs 1995), the diversity of ways it can be manipulated *in vitro* (Bajaj 1987) and problems that are amenable to genetic engineering (Belknap *et al.* 1994) it is a favoured plant for applied biotechnology.

A variety of transformation systems have been used with potato including polyethylene glycol induced uptake (Fehér *et al.* 1991), electroporation (Masson *et al.* 1989), *A. rhizogenes* (Ooms *et al.* 1986) and *A. tumefaciens* (for example Stiekema *et al.* 1988). *Agrobacterium* based systems are by far the most frequently used for potato and transgenic plants have been produced using a range of explants including mature tubers (Sheerman and Bevan 1988), *in vitro* microtubers (Ishida *et al.* 1989), leaf discs (de Block 1988) and internodes (Newell *et al.* 1991). In addition to *Solanum tuberosum*, a number of other *Solanum* species have been transformed including *S. commersonii* (Cardi *et al.* 1992), *S. integrifolium* (Rotino *et al.* 1992), *S. brevidens* (Annie Liu *et al.* 1995), *S. demissum, S. hjertingii, S. papita, S. stoloniferum* and *S. verrucosum* (Kumar *et al.* 1995) which should be useful for antibiotic selection of somatic hybrids and gene isolation.

Potato plants have been produced with resistance to a number of important virus diseases including PVX (Xu *et al.* 1995), PVY (Farinelli *et al.* 1992) and resistance has been confirmed in field trials (Kaniewski *et al.* 1990; Lawson *et al.* 1990; Jongedijk *et al.* 1992). PLRV is a major, world-wide virus disease of potato and can be difficult to control as it is aphid transmitted.

There are sources of natural resistance to PLRV in wild relatives of the domesticated potato. The best known is *Solanum brevidens*, but it is sexually incompatible with potato and alternative approaches such as protoplast fusion must be used (Austin *et al.* 1985). However, introduction of these resistance genes would require many years, perhaps decades of extensive breeding. Introduction of viral genes into the genome of plants by, for example, *Agrobacterium* mediated transformation has been shown to be an effective mechanism for obtaining viral resistance (Baulcombe 1996). This approach provides a rapid method of introducing virus resistance to elite cultivars and parental lines without disturbing their genetic makeup.

The nucleotide sequence of various isolates of PLRV have been determined (Keese *et al.* 1990) and the coat protein gene introduced into a number of potato cultivars including Desiree (Kawchuk *et al.* 1990), Ranger Burbank and Russet Burbank (Brown *et al.* 1995) and Atlantic and Kennebec (Graham *et al.* 1995). Further study at CSIRO, Division of Plant Industry has identified a construct (pG10) which gives good protection against PLRV. A construct based on the PLRV polymerase gene has also been produced. The attraction of

the pRolCpol construct is that it appears to be free from intellectual property restrictions. However, its efficacy at protecting plants from PLRV has not been evaluated.

Research presented in this section utilises and integrates data generated from the two previous sections to produce a population of transgenic plants. The cultivars Crystal, Sebago D, 80-90-5 and 80-93-4 have been transformed with pG10 and the cultivars Crystal and 80-90-5 have been transformed with pRolCpol.

4.2 Methods and materials

4.2.1 General considerations

Experiments were done using the cultivars Crystal, Sebago D (a clone of Sebago extensively grown in Queensland), 80-90-5 and 80-93-4. Each cultivar was maintained in tissue culture as described in section 2.2.2.

4.2.2 Gene constructs and preparation of *Agrobacterium*

The construct pG10 (Fig. 1) uses the CaMV 35S promoter to express PLRV coat protein. Within the 3' untranslated region of the transgene mRNA are two ribozyme (gene shear) domains targeting the 5' end of the PLRV genome. The extreme 3' end of the transgene mRNA contains a replicator sequence which is designed to cause the production of a complementary strand of the mRNA in the presence of the virus. The PLRV coat protein and ribozymes have been cloned into pJ35CN (Walker *et al.* 1987) and the entire plasmid cloned into the T-DNA from pGA470 (An *et al.* 1985).

The pRolCpol construct (Fig. 2) uses the *RolC* promoter, which results in vascular specific expression in potatoes (Graham *et al.* 1997). The construct expresses the carboxy-terminal open reading frame of the PLRV polymerase.

Two control constructs were also used with Sebago D; pS4GUS (Fig. 3), which expresses the *gus* gene (Section 3.1) and pPPO which knocks out endogenous polyphenoloxidase in potatoes.

Each construct was transferred to *Agrobacterium* strain LBA 4404 as described in section 3.2.2.

For transformation, *Agrobacterium* cultures were grown on LB medium with appropriate antibiotics on 9 cm Petri plates for 48 hrs at 26 °C. Cells were flushed off the Petri plates with 20 mL Murashige and Skoog (1962) medium with 30 gL^{-1} sucrose (MS) and diluted to a cell density of 2. 10^5 cells mL^{-1}.

4.2.3 Regeneration and transformation

The callus initiation media required for plantlet regeneration for each cultivar have been previously described (Table 1i).

Transformations were performed by placing a potato shoot in the *Agrobacterium* solution and preparing 1 cm internode explants. These were blotted dry and transferred to co-cultivation medium (MS with 100 µM acetosyringone) for three days. Internodes were then transferred to pre-selection medium (appropriate callus initiation medium with 150 µg mL^{-1} timentin [or 250 µg mL^{-1} cefotaxime]) for seven days after which they were transferred to selection medium (regeneration medium with timentin [or cefotaxime] and 50 µg mL^{-1} kanamycin). Explants were transferred to fresh selection medium at 21 d intervals until shoots developed. Regenerated shoots were transferred to MS medium with timentin [or cefotaxime] and 50 µg mL^{-1} kanamycin for root initiation and routine maintenance. Regenerated shoots that initiated roots were considered to be putative transformants and subjected to more detailed biochemical and molecular analysis. Details of the standardised transformation protocol are provided in Appendix 3.

4.2.4 Analysis

4.2.4.1 Polymerase Chain Reaction

Plants that initiated roots in media with 50 µg mL^{-1} kanamycin were screened for the presence of the *npt II* gene. DNA was extracted from leaf tissue (Fulton *et al.* 1995) and quantified fluorometrically (Hoefer TKO 100). PCR amplifications were done in a 50 µL reaction mix comprising 50 ng DNA, 100 ng of each primer, 100 µM of dNTPs, 3 mM $MgCl_2$, 10 mM KCl, 100 mM Tris (pH 8) and 1U polymerase (Ampli*Taq*, Perkin Elmer). The sequence of primer 'NPT 1' was 5' - GAG GCT ATT CGG CTA TGA CTG - 3' and for primer 'NPT 2' was 5' - ATC GGG AGC GGC GAT ACC GTA - 3', which amplifies a 700 bp fragment. DNA was amplified through 33 cycles of 20 sec at 95 °C, 20 sec at 55 °C and 1 min at 72 °C after which 25 µL of reaction product was separated by electrophoresis through a 1% agarose gel with x 1 TAE buffer.

4.2.4.2 NPT II dot blot assay

The activity of NPT II was determined essentially as described by Mc Donnell *et al.* (1987).

4.2.4.3 Southern analysis

Total genomic DNA was extracted from leaf tissue, digested with *Bgl* I blotted to Hybond N^+ membrane and hybridised with radiolabelled PLRV coat protein derived cDNA using methods essentially described in Sambrook *et al.* (1989). Approximately 15 µg of DNA was loaded per lane. Uncut plasmid containing the PLRV coat protein sequence was also loaded at concentrations equivalent to 10, 5, 2 and 1 gene copy per 15 µg of total genomic DNA. The washed membrane was analysed using a phosphoimager.

4.2.5 Glasshouse studies

4.2.5.1 Aphid challenge

Plants were acclimatised using standard procedures and grown in a PH-1 containment glasshouse. Aphids (*Myzus persicae*) were maintained on virus-free Chinese cabbage. For virus inoculation, aphids were fed on PLRV-infected *Physalis floribunda* for three days then caged onto test plants for a three to seven day inoculation period (*ca.* 15 aphids per plant). The aphids were subsequently killed by watering the plants with Folimat.

4.2.5.2 ELISA

Three weeks after inoculation leaf samples were collected and analysed with ELISA for PLRV using a Sanofi Plantest Kit according to the manufacturers instructions. Plants known to be infected with PLRV and others known to be PLRV-free were always included as controls. Usually at least three replicates were used per sample. Ten replicates of each transgenic line were tested.

4.3 Results

4.3.1 Regeneration and transformation

A total of 12,394 internode explants from the four cultivars were processed with the two anti-viral gene constructs and 96 putative transgenic plants were produced using 50 μg mL^{-1} kanamycin as a selection pressure (Table 13). An additional 600 explants of Sebago D were inoculated with pS4GUS or pPPO and 35 putative transgenic plants regenerated.

Plants have been regenerated, with varying degrees of success from each of the four cultivars and gene constructs with the exception of Sebago D and 80-93-4 with pRolCpol where no plants were obtained. It was apparent from this data that for each of the four cultivars that more plants regenerated when explants were inoculated with pG10. The nature of the of the cultivar-gene construct interaction was elucidated by analysis of the data. Data were analysed by expressing the number of putative transgenic plants as a percentage of the theoretical number of shoots that could have been regenerated if no transformation or selection had been imposed. This is the product of the regeneration index (the number of shoots that can be regenerated from 100 explants) for each cultivar (Table 1j) and the number of explants processed (Table 13) divided by 100.

For the anti-viral constructs, more shoots were regenerated, irrespective of cultivar, if explants were co-cultivated with pG10 than with pRolCpol (Table 14). The constructs pS4GUS and pPPO were only tested with Sebago D and the transformation efficiency is superior to the anti-viral constructs. Plants transformed with pS4GUS expressed the *gus* gene in leaf, stem and tubers (Fig. 4).

Presently we have a population of 96 plants resulting from co-cultivation with the anti-viral constructs that have initiated roots in the presence of 50 μg mL^{-1} kanamycin. These are

being assessed further using a range of techniques such as PCR, dot blot assays, Southern analysis and challenge with viruliferous aphids in glasshouse experiments.

Not all the putative transgenic plants have been assessed and data that follows is representative of analysis to date.

4.3.2 Polymerase Chain Reaction

Plants can be readily assessed for the presence of introduced genes using PCR. Results from screening 13 lines of 80-90-5 co-cultivated with pG10 that were selected at random from those that initiated roots on medium with 50 $\mu g\ mL^{-1}$ kanamycin, found that nine contained the expected 700 bp fragment (lanes 2, 3, 4, 7, 8, 11, 12, 13 and 14) and one had two bands, one at 600 bp and another at 1.4 kb (lane 5) (Fig. 5).

4.3.3 NPT II dot blot assay

Four lines of Sebago D co-cultivated with pG10 were analysed for NPT II activity. The blot (Fig. 6) shows that two of the lines (#1 and #2) have high levels of NPT II activity, whereas two other lines (#3 and #4) have little detectable activity and are either non-transgenic or have had their NPT II genes silenced.

4.3.4 Southern analysis

Genomic DNA extracted from Sebago D line #1 and #2 was analysed for the PLRV coat protein/ribozyme/replicator sequence. The number of copies of the transgene can be determined by the number and intensity of the bands in the autoradiograph (Fig. 7). Line #1 has two bands, the top band has the intensity expected of one copy, whereas the bottom band has the intensity expected of two copies. Therefore #1 probably contains three copies of the G10 genes. Line #2 has two bands of the intensity expected for a single copy and therefore probably contains two copies of the G10 gene in its genome.

4.3.5 Glasshouse studies

Three weeks after inoculation with PLRV Sebago D lines #1 and #2 had PLRV levels less than non-transgenic or *Physalis floribunda* plants (Fig. 8). Line #1 had the lowest level but both lines look sufficiently promising to merit field trials.

Of the 14 lines of 80-90-5 co-cultivated with G10, line #7 shows no PLRV resistance, lines #1 and #18 showed moderate to weak resistance. The other 11 showed high levels of resistance or immunity to PLRV (Fig. 9) but the possibility exists that they are not resistant or immune but missed PLRV infection by aphids not feeding on the plant. They look extremely promising and should be retested.

4.4 Discussion

A population of transgenic potato plants which contain genes the control PLRV has been produced. They represent four cultivars that are commonly used as parental lines in the fresh market component of NaPIES. In addition, two of the cultivars, Crystal and Sebago are commonly grown for the fresh market.

The differences in the transformation efficiency of the gene constructs is difficult to explain. It cannot be due to the *Agrobacterium* strain as all constructs are in LBA 4404 and have the same *vir* genes. There is only a limited amount of information where different gene constructs have been compared in the same *Agrobacterium* strain on transformation efficiency and the results are variable. With Russet Burbank one report found no difference between binary and co-integrate vectors in the strain C58C1 (de Block 1988) whereas with anti-viral constructs for PVX and PVY in an unnamed *Agrobacterium* strain there was a difference (Newell *et al.* 1991).

Selection of putative transgenic shoots and rooting with 50 µg mL^{-1} kanamycin is effective. At this concentration kanamycin is phytotoxic and prevents regeneration of adventitious shoots and roots.

Screening plants with PCR or a dot blot assay is a rapid and reliable method to determine if material is transgenic. Plants that initiated roots in the presence of kanamycin and were negative when screened with PCR (Fig. 4, lanes 6, 9 and 10) could be chimeric. The plant in lane 5 which has a unexpected band at 1.4 kb could have two copies of the *npt II* gene next to one another.

Southern analysis confirmed that PLRV coat protein/ribozyme/replicator sequence was stably integrated in the genome. Gene insertion is a random event and it common for more than one copy of the gene of interest to be inserted.

Lines of Sebago and 80-90-5 with promising levels of resistance to PLRV should undergo field trials. An application will be submitted the the Genetic Manipulation Advisory Committee and depending on the result of the application and funding, this will proceed in 1997. A PLRV-resistant Sebago line should be well received in Queensland where Sebago is widely grown and PLRV a major disease. In addition the transgenic lines can be incorporated into the conventional breeding programme in NaPIES.

Since gene transfer was first demonstrated to be a practical alternative to conventional breeding 12 years ago the developments have been rapid and spectacular. A range of genes have been introduced to a variety of plant species and products have already been commercialised. In 1994 the first transgenic plant product, the Flavr Savr™ tomato (which shows reduced fruit softening), was commercialised, only 10 years after gene transfer systems were developed. Six other transgenic crops were approved for commercial release the following year (cotton, soybean, maize, canola, potato and squash) with traits including herbicide, insect and virus resistance (Redenbaugh *et al.* 1996). There have now been over 3,467 field trials (January 1996) of transgenic plants conducted in 34 countries and these

have included 40 different plant species. This explosion of transgenic crops will be an increasingly familiar sight to both farmers and consumers.

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Table 13. Summary of the number of explants processed and putative transgenic plants regenerated with four cultivars after inoculation with four gene constructs

Cultivar	Gene construct	Explants [1]	Plants [2]
Crystal	pG10	847	14
	pRolCpol	3,134	1
Sebago D	pG10	1,700	9
	pRolCpol	1,100	0
	pS4GUS	240	10
	pPPO	360	25
80-90-5	pG10	612	55
	pRolCpol	2,801	4
80-93-4	pG10	1,400	13
	pRolCpol	800	0

[1] Number of internode explants processed.

[2] Putative transgenic plants regenerated after selection and root initiation with 50 μg mL^{-1} kanamycin.

Table 14. Effect of gene construct on transformation efficiency for each cultivar [(1)]

Cultivar	Gene construct			
	pG10	pRolCpol	pS4GUS	pPPO
Crystal	0.3	0.006	nt[(2)]	nt
Sebago D	0.07	0	0.5	0.9
80-90-5	1.3	0.02	nt	nt
80-93-4	0.1	0	nt	nt

[(1)] The figures represent the number of shoots regenerated for each cultivar and gene construct (Table 13) expressed as a percentage of the number of shoots that theoretically could have been regenerated if no transformation or selection had been imposed. The theoretical shoot number is the product of the regeneration index for each cultivar divided by 100 (Table 1j) and the number of explants processed (Table 13)

[(2)] not tested

Figure 1. Map of gene construct pG10

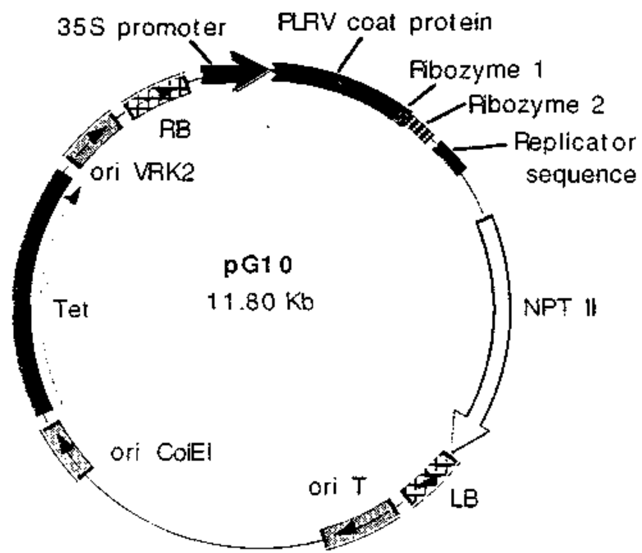


Figure 2. Map of gene construct pRolCpol

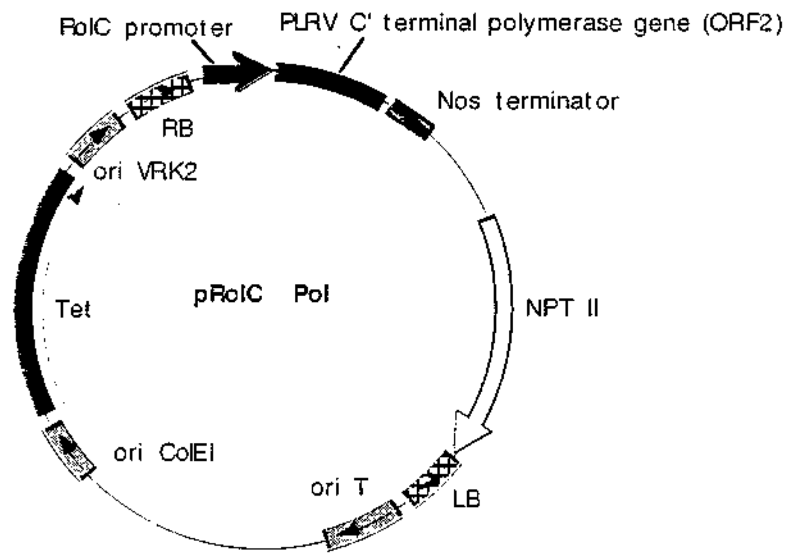


Figure 3. Map of gene construct pS4GUS

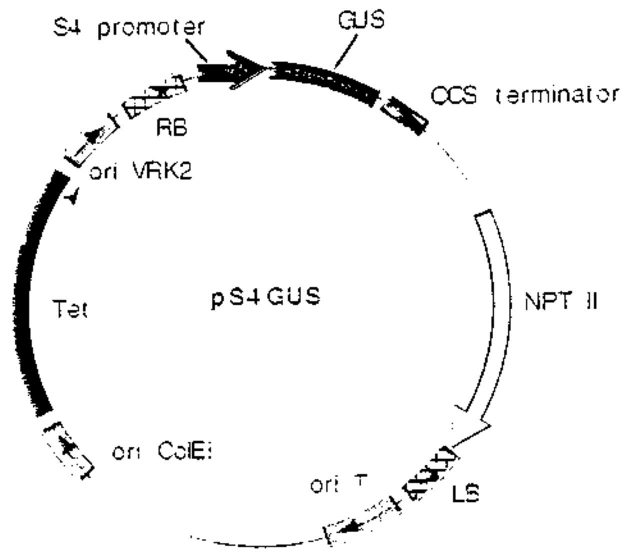
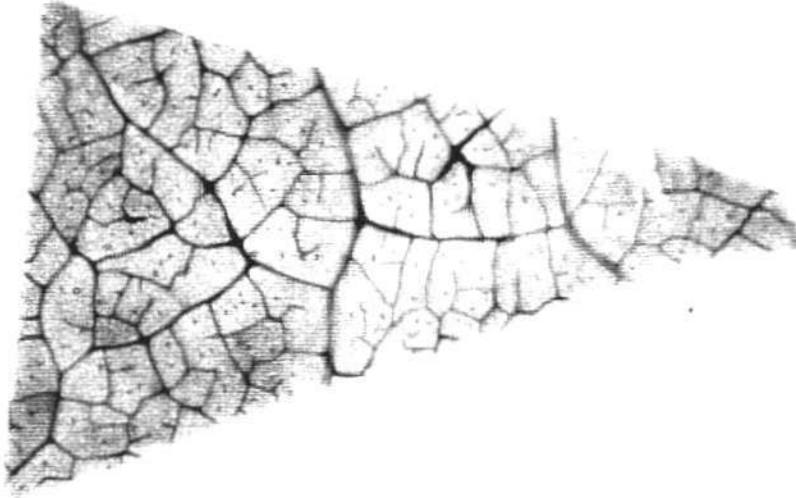
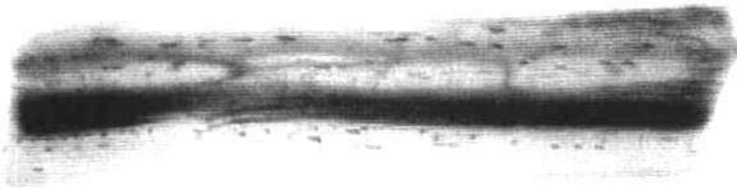


Figure 4. GUS expression in leaf, stem and tuber in Sebago transformed with pS4GUS

Leaf



Stem



Tuber

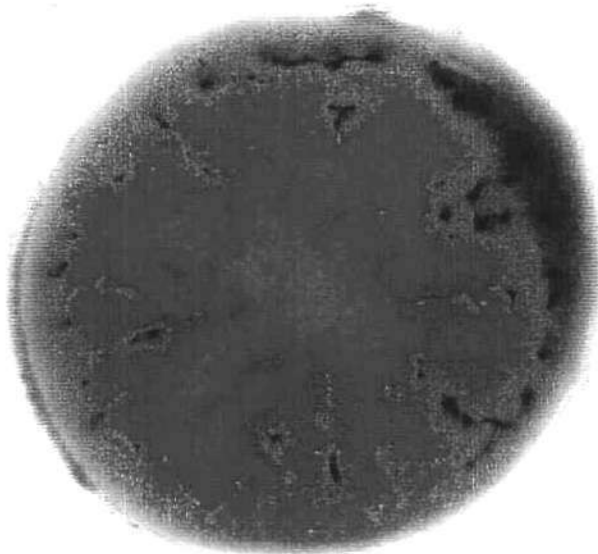
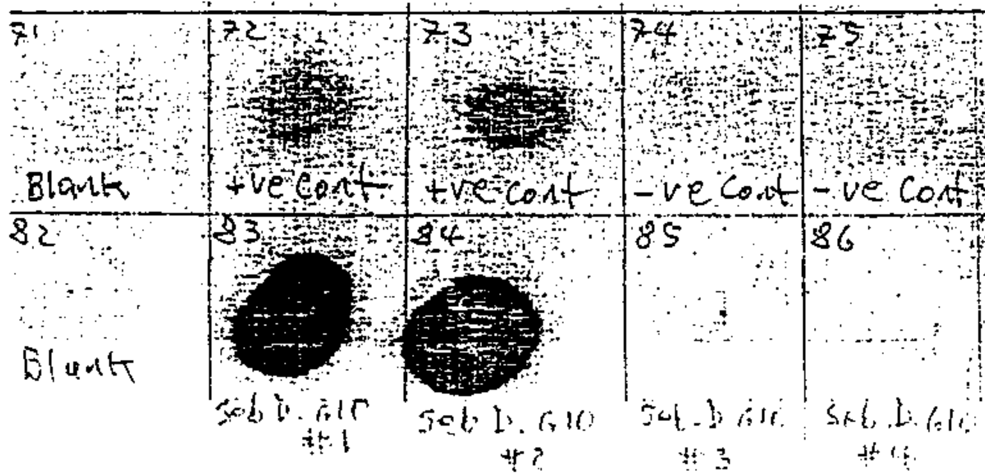


Figure 5. Agarose gel electrophoresis of a PCR-amplified *npt II* sequence from 80-90-5 plants co-cultivated with pG10

100 = 100 bp marker (the brightest band [arrowed] is 800 bp). Lane 1 is a negative control and lanes 2 to 14 are putative transgenic plants

Figure 6. NPT II dot blot assay of Sebago D leaf extracts co-cultivated with pG10



71 and 82 = blank i.e. no plant extract

72 and 73 = positive controls i.e. plants known to be expressing NPT II

74 and 75 = negative controls i.e. plants not co-cultivated

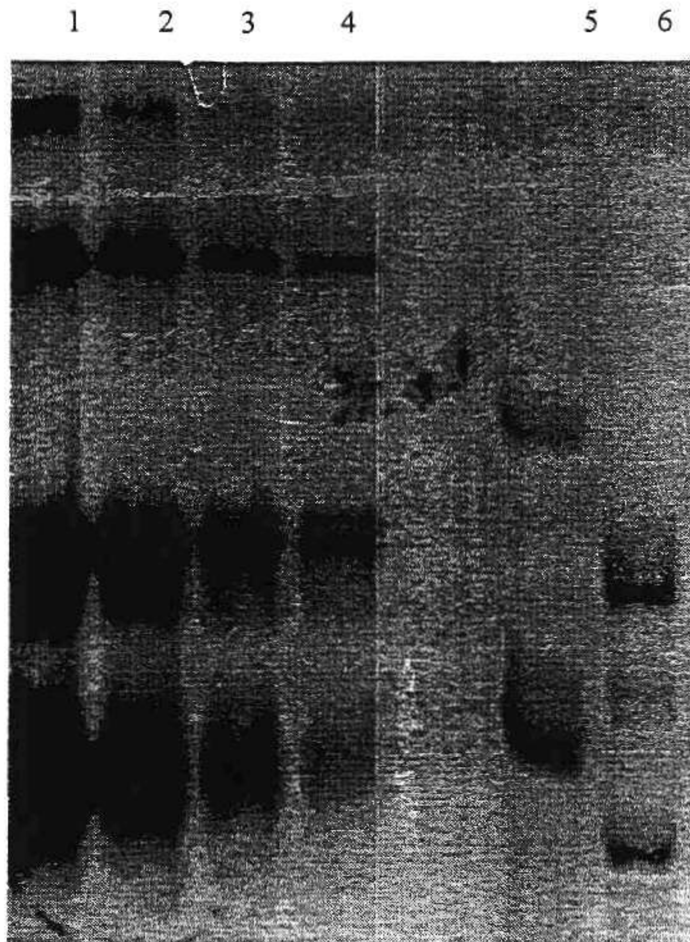
83 = Line #1

84 = Line #2

85 = Line #3

86 = Line #4

Figure 7. Southern blot analysis of Sebago D co-cultivated with pG10

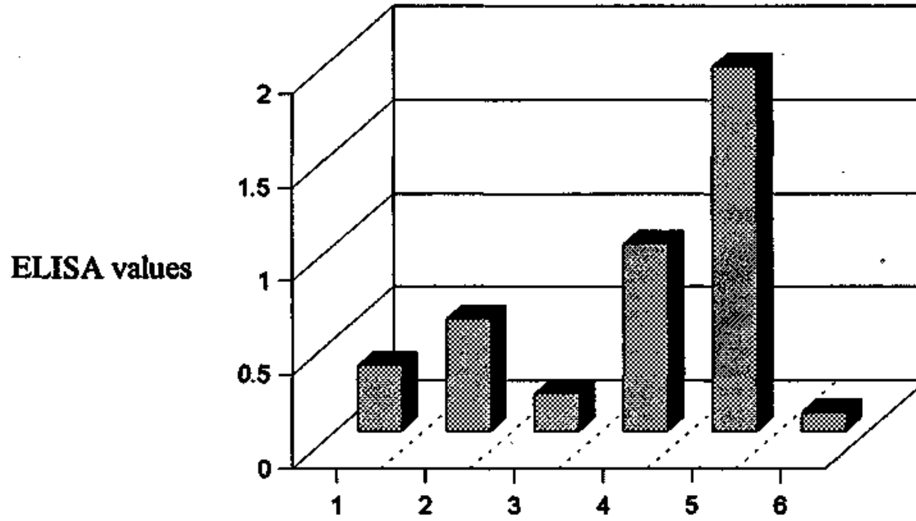


Lanes 1, 2, 3 and 4 = 10, 5, 2 and 1 gene copy

Lane 4= Sebago D #1

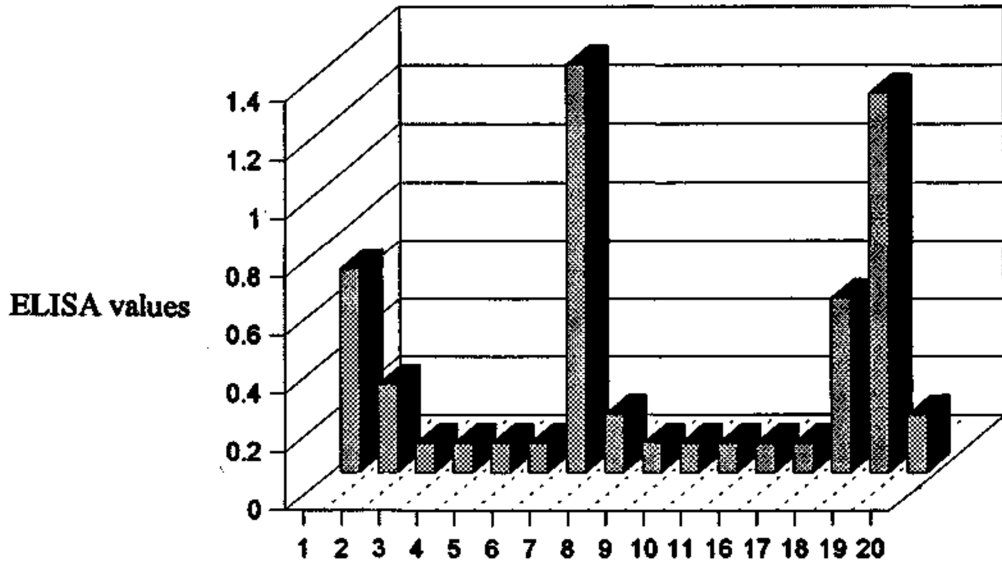
Lane 5 = Sebago D #2

Figure 8. PLRV level in Sebago D plants co-cultivated with pG10



1 = Sebago #1
2 = Sebago #2
3 = Sebago negative control
4 = Sebago positive control
5 = *Physalis*
6 = Buffer

Figure 9. PLRV level in 80-90-5 plants co-cultivated with pG10



1 to 18 are 80-90-5 plants co-cultivated with pG10
19 = positive control
20 = negative control

5.0 Recommendations

5.1 Extension and adoption by industry

The results of this research describe the production of a population of transgenic plants that contain genes to control PLRV. Prior to this material being used by industry it is necessary for it to be evaluated in glasshouse and field trials, after which it will have two uses.

Firstly it can be incorporated into the conventional breeding programme so that this difficult to breed for characteristic can be sexually transmitted. Secondly the cultivars Crystal and Sebago can be commercialised as new cultivars.

An application to develop these ideas will be submitted for funding.

5.2 Directions for future research

The areas recommended for further research are;

- confirm the results obtained with Sebago and 80-90-5 with additional glasshouse experiments,
- screen the remaining transgenic plants for response to PLRV infection,
- screen selected lines of Crystal and Sebago D in field trials for agronomic performance with the aim of eventual release to growers,
- determine the sexual transmission of the anti-viral genes through hybridisation to other cultivars and incorporate selected lines into the conventional breeding programme,
- generate additional transgenic lines with new gene constructs for PLRV and PVY in new binary vectors (see 5.3 below).

5.3 Financial/commercial benefits of adoption of research findings

The financial benefits from this research will occur in two ways. Firstly as a result of increased yields due to PLRV resistance and secondly from not having to apply insecticides to control aphids. The extent of these benefits is impossible to determine at present.

The commercial release of these lines or derivates, however will require licencing agreements from other agencies. Such negotiations are currently in progress with Monsanto for the commercial production of Kennebec in Australia and if an agreement can be reached this could probably be extended to cover cultivars used in this project.

Recently the CSIRO, Division of Plant Industry and CRC for Plant Science have developed virus resistance genes for PLRV and PVY, a selectable marker cassette and a binary vector. All these elements appear to be effective and free of intellectual restrictions. This is currently being checked by European patent attorneys. With these advances and the regeneration and transformation procedures developed in this project, it should now be possible to produce virus resistant potato cultivars for unimpeded introduction into Australian agriculture.

Appendix 1. Nominated cultivars and there attributes

Coliban	most commonly used parent high yield smooth bright white skin
Crystal	outstanding skin colour good inheritability
Sebago	extensively grown in Queensland where PLRV is a major problem
Wilwash	well adapted to Riverland\Riverina
66-11-2	tends to produce a high yield uniform tubers white skin
80-90-5	high resistance to powdery scab with good inheritability of characteristic red and white smooth skin
80-93-4	high yield red skin
87-12-6	red skin
87-57-9	red skin

Appendix 2. Conditions for adventitious shoot regeneration

	Callus initiation	Regeneration
Medium	various (see Table 1i)	MS with 10 µM BAP and 15 µM GA_3
Duration	7 days	55 days
Incubation conditions	22 °C 16:8 hr (light:dark) 25 µM m^{-2} sec^{-1}	22 °C 16:8 hr (light:dark) 75 µM m^{-2} sec^{-1}

Appendix 3. Standardised transformation protocol

Day	Activity	Comment
0	Prepare *Agrobacterium* inoculum Inoculate internode explants Transfer to co-cultivation medium	MS with 100 μM acetosyringone
3	Transfer to pre-selection medium	Preferred callus initiation medium (see Table 1i) with 100 μg mL^{-1} timentin [or 250 μg mL^{-1} cefotaxime]
10	Transfer to selection medium	Regeneration medium (see Appendix 2) with timentin [or cefotaxime] with 50 μg mL^{-1} kanamycin.
31	Transfer to selection medium	
52	Transfer to selection medium	Adventitious shoots can be transferred to MS medium with timentin [or cefotaxime] with 50 μg mL^{-1} kanamycin.

Appendix 4. Publication arising from this research

Hutchinson, J.F., Moran, J., Androas, A., Graham, M.W. and Waterhouse, P. (1995). Biotechnology and potato improvement. National Potato Growers' Field Day, National Potato Improvement Centre-Toolangi. pp. 3.

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Hutchinson, J.F., Gumley, S., Kariuki, A., Moran, J., Barlass, M., Robinson, S., Graham, M.W. and Waterhouse, P. (1996). No sex please - we're genetic engineers. *Potato Australia* 7, 18-19.