Know-how for Horticulture™

Improved carrot and celery cultivars through biotechnology

Dr Gowri Maheswaran
VIC Department of Primary Industries

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Level 1, 50 Carrington Street
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T: (02) 8295 2300
F: (02) 8295 2399

E: info@horticulture.com.au

Production and assessment of virus resistant carrot and celery cultivars for the fresh market

Final report for Horticulture Australia project VG01043

Prepared by

Nina McCormick, Rebecca Ford and Brendan Rodoni

Department of Primary Industries, Knoxfield

and

The University of Melbourne

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Principle investigators:
Nina McCormick
Faculty of Land and Food Resources
The University of Melbourne
Royal Parade
Parkville
VIC 3010
Ph. (03) 8344 5019
Fax. (03) 98908098
Email: n.mccormick@pgrad.unimelb.edu.au

Brendan Rodoni
Primary Industries Victoria
Knoxfield
Private Bag 15
Ferntree Gully Delivery Centre
VIC 3180
(03) 9210 9222
(03) 9800 3521
brendan.rodoni@dpi.vic.gov.au

Rebecca Ford
Faculty of Land and Food Resources
The University of Melbourne
Royal Parade
Parkville
VIC 3010
Ph. (03) 8344 5019
Fax. (03) 98908098
Email: rebeccaf@unimelb.edu.au

Project Goals:
This project (VG07798) was directed towards:

1. The development of tissue culture systems for the efficient regeneration of transgenic carrot and celery somatic embryos from petiole tissue.

The development of *Agrobacterium* transformation systems for the efficient transfer of anti-viral genes into carrot and celery tissue.

2. The isolation and characterisation of a suitable short sequence of Carrot virus Y and Celery mosaic virus for integration into intron-containing hairpin RNA antiviral constructs.
3. The production of a population of transgenic carrot and celery with anti-viral genes, the establishment of disease challenge methods and the preliminary assessment of transgenic carrot and celery lines transformed with anti-viral genes.

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1.0 Introduction

Many crops are susceptible to infection by plant viruses, particularly potyviruses such as *Carrot Virus Y* (CarVY) and *Celery mosaic virus* (CeMV), and can cause serious damage to the plants they infect, and in turn reduce the economic value of a crop to the grower. The Potyvirus genus is the largest taxon of plant viruses and includes many economically important species. Many attempts to control or prevent infection of a crop by potyviruses have been ineffective due to the highly efficient transmission of these viruses by aphids and the presence of alternate hosts in and around the affected crop.

Conventional measures such as crop rotation, early detection, destruction of infected source plants and chemical control of insect vectors have been used to control viral pathogens. For the control of CarVY and CeMV, the carrot and celery industries recommend planting tolerant varieties together with cultural crop management measures including carrot-free growing periods. However, conventional breeding for CarVY and CeMV resistance is not possible due to a lack of naturally available resistance sources. To further compound this problem the tolerance/susceptibility of carrot and celery cultivars to these viruses is not clearly understood.

Increased knowledge of the molecular genetics of plant viruses and their hosts' natural defence mechanisms has resulted in novel methods to control virus diseases in plants. Enabling carrot and celery cultivars to recognise invading virus and marshal internal defences will equip plants with endogenous protection, thereby reducing crop losses and promoting sustainable production systems.

This report summarises research conducted by the Department of Primary Industries, Knoxfield and The University of Melbourne to use gene technology to produce carrot and celery cultivars resistant to *Carrot virus Y* (CarVY) and *Celery mosaic virus* (CeMV) respectively. This report has been divided into four main sections:

1. The development of a suitable regeneration system for the production of transgenic plants (Chapters 2 and 3).
2. The development of a robust and efficient transformation system to deliver foreign DNA to cells (Chapter 4).
3. The isolation and characterisation of suitable viral genes that can be used to confer resistance (Chapter 5).
4. The production and screening of a population of transgenic plants with potential virus resistance genes (Chapter 6).

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1.1 Media Summary

Improved carrot cultivars with resistance to important virus diseases have been generated using gene technology

Carrot virus Y (CarVY) is a virus that infects carrot, causing foliage mottle, plant stunting and distorted roots. First seen in Western Australia in 1997, CarVY has now infiltrated all carrot growing regions of Australia and is fast becoming a growing concern for Australian carrot producers. Infection incidences of up to 95% have been reported and yield loss above 35% have cost the Australian carrot industry between $5 and $10 million a year. CarVY is transmitted in the field by aphids and insecticides for aphid control are largely ineffective. The virus is currently controlled by employing a 'carrot-free' growing period that removes or reduces the source of virus infection.

Conventional breeding for CarVY resistance is not possible due to a lack of naturally available resistance sources, therefore alternative approaches need to be explored. One such strategy is the application of RNA interference (RNAi) technology, which offers an alternative and practical approach to developing virus resistant carrot cultivars. This evolutionary conserved plant defence mechanism enables the plant to subsequently recognise invading virus.

Researchers from the DPI-Knoxfield, The University of Melbourne and CSIRO have been collaborating for the last 10 years and have developed ways to use RNAi technology to produce virus resistant carrot cultivars. In this project, a population of transgenic lines of the cultivar Crusader has been produced with anti-viral genes.

These lines have been assessed in glasshouse trials, where plants have been inoculated with virus and their response monitored. Plants with resistance to CarVY have been identified and are ready for field trials to assess agronomic performance and virus resistance under field conditions. Enabling carrot cultivars to recognise invading virus and marshal internal defences will equip plants with endogenous protection, thereby reducing crop losses and promoting sustainable production systems.

1.2 Technical Summary

Carrot virus Y (CarVY) and *Celery mosaic virus* (CeMV) are the most significant viral pathogens of carrot and celery in Australia. While CarVY is found only in Australia, infection incidences of up to 95% and yield loss above 35% have cost the Australian carrot industry between $5 and $10 million a year (Latham and Jones 2000; Latham and Jones 2004). CeMV was first reported in celery plants in California in 1938 and is now thought to be distributed over most celery growing regions of the world. Initial reports of CeMV in Australia occurred in South Australia in 1985 and subsequently, the virus has spread to all celery growing districts and has been detected in Australian carrots (Alberts *et al*. 1989; Traicevski *et al.* 1999). Both viruses are transmitted in the field by aphids and insecticides for aphid control are largely ineffective. The virus is currently controlled by employing a 'carrot/celery free' growing period that removes or reduces the source of virus infection.

There is no known natural source of resistance to CarVY and limited natural sources of CeMV resistance that can be easily transferred to carrot and celery cultivars. RNA interference (RNAi), a form of gene technology, presents an alternate and practical approach to developing virus resistant carrot and celery cultivars. RNAi technology enables the transgenic plant to recognise the invading virus and trigger conserved plant defence mechanisms that will destroy the genetic material of the invading virus.

There are four steps required in delivering RNAi technology to a plant species:

- The production of a suitable regeneration system for the production of transgenic plants.
- The development of a robust and efficient transformation system to deliver foreign DNA to cells.
- The isolation and characterisation of suitable viral genes that can be used to confer resistance.
- The production and screening of a population of transgenic plants with potential virus resistance genes.

A suitable tissue culture regeneration system has been developed for the production of transgenic carrot cultivars Stefano and Crusader and celery cultivars Summit and Tendercrisp. A robust and efficient transformation system to deliver foreign DNA to cells has also been developed. Suitable CarVY and CeMV genes that can impart resistance have been characterised and cloned. These genes have been incorporated into new and improved gene constructs to provide resistance to CarVY and CeMV in carrot and celery cultivars respectively.

A population of transgenic plants of carrot cv. Crusader has been produced and glasshouse screening has identified a number of plants with resistance to CarVY. These selections are ready for small-scale field trials to assess agronomic performance and virus resistance under field conditions.

The development of suitable regeneration and gene transformation systems for carrot and celery is of significant value for industry. In this project we have targeted virus resistance genes to be incorporated into carrot and celery to confer resistance as a model system. However any gene technology approach can be used to enhance the

properties of carrot and celery using the regeneration and transformation systems developed in this project.

1.3 References

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Traicevski, V., Schreurs, T., Rodoni, B. and Moran, J. (1999) Celery mosaic potyvirus occurring naturally in cultivated Apiaceae in Victoria, Australia, *Australasian Plant Pathology*, 28:92.

2.0 Tissue culture and regeneration of *Daucus carota* var. *sativum* cultivars Stefano and Crusader

2.1 Introduction

Nearly 50 years ago researchers first demonstrated that aseptically cultured carrot cells could give rise to embryo-like structures, from which entire plantlets could form (Reinert 1958; Steward *et al.* 1958; Reinert 1959). The carrot regeneration system is now so well known that it is referred to as the model for somatic embryogenesis (Smith and Krikorian 1989). Somatic embryogenesis in carrot cells was initially developed as a research tool to investigate physiological and biochemical aspects of carrots and plants in general (Fujimura *et al.* 1980; Kamada and Harada 1981; Nomura and Komamine 1986b; Smith and Krikorian 1989; Wake *et al.* 1995; Tsukahara and Komamine 1997) and continues to be used to explore complex biochemical processes within plant cells (Ibaraki *et al*. 2000; Imani *et al.* 2001; Jiménez and Bangerth 2001).

As well as the exploration of carrot somatic embryo development, the system has been used for *in vitro* production of artificial seeds (Osuga and Komamine 1994), rapid plant propagation (Fujimura and Komamine 1979; Vasil 1984; Osuga and Komamine 1994) and crop improvement (Nomura 2003). The *in vitro* culture of cells is becoming increasingly important for maintaining transgenic plant lines (Nomura 2003).

Methods for initiating and maintaining cell and asexual embryo cultures are well established (Halperin and Wetherell 1964; Fujimura and Komamine 1975; McWilliam *et al*. 1975; Kamada and Harada 1979). Somatic embryogenesis in carrot can be readily induced by the transfer of callus cells cultured in an auxin-containing medium to an auxin-free medium (Halperin and Wetherell 1964; Kamada and Harada 1981; McWilliam *et al*. 1974; Ozeki and Komamine 1981; Borkird *et al*. 1988; Komamine *et al*. 1992; Nissen and Minocha 1993; Nomura 2003). In this chapter, the effects of varying concentrations of 2,4-D on callus initiation from seedling- and petiole-derived tissue for optimal callus proliferation of carrot cultivars Stefano and Crusader were explored. The effects of removing auxin or the addition of cytokinin to plant growth medium on the regeneration capacity from callus explants was also investigated.

No reports exist for clonal propagation from basal meristematic material of carrot plants. Micropropagation using meristematic proliferation can be achieved in a relatively short time for the maintenance of transgenic or non-transgenic clonal lines if the carrot tissue can be prevented from reverting to an undifferentiated state as is indicative of somatic embryogenesis. To determine which plant growth regulator (PGR) treatments may be successfully applied for micropropagation of carrot plants, the system for clonal propagation of strawberry plants was assessed. Strawberries have a growth physiology similar to that of carrot, with the meristematic material found at the base of plants at the juncture of petioles and roots. The PGR treatment most commonly used for micropropagation of strawberry plants was tested for its ability to multiply carrot plants from meristematic material.

2.2 Materials and methods

2.2.1 Plant materials

Carrot (*Daucus carota* var. *sativum*) seeds of cvs. Stefano, Crusader, Kendo, Murdoch and Omeros (South Pacific Seeds Pty Ltd, Australia) were surface sterilised in 70% ethanol containing 0.01% Tween 20 for 1 min and in 2% available chlorine and 0.01% Tween 20 for 20 min with shaking. Seeds were washed three times in sterile deionised water, then germinated in sterile tubs for three weeks on agar-solidified, hormone-free MS salts and vitamins (Murashige & Skoog 1962) at 25ºC with a 16h/8h light/dark photoperiod. For the assessment of seed viability and control of microbial contaminants to ensure adequate seedling plant material for initial callus initiation experiments, a germination trial was conducted with six repetitions of 20 seeds each. The non-contaminated germinated seeds were calculated as a percentage of the number of seeds germinated at day 7 following placement of seed on germination medium.

Following germination, seedlings were either used as a source of explant material for callus initiation experiments or as a source of meristematic shoot tips for stock plant culture. For shoot tip growth, carrot meristematic tips were transferred to hormone-free MS medium. Stock plants were maintained on hormone-free MS medium, routinely subcultured every eight weeks and used as a source of explant material for subsequent callus initiation experiments. Subculture of stock plants involved the removal of roots and callus material from below the meristematic base and removal of the older petiole and leaf material from the meristematic base leaving 2-3 young, actively growing petioles.

2.2.2 Micropropagation

For the purpose of stock plant proliferation, stock plants of carrot cultivars Crusader and Stefano were subcultured (2.2.1) and placed on treatment medium comprising MS salts and vitamins with the plant growth regulator (PGR) 5 µM 6-benzylaminopurine (BAP). A control, comprising of subcultured stock plants placed on agar solidified MS without PGR, was used for comparison with the treatment medium. For micropropagation experiments, plants were maintained at 25ºC with a 16h /8h light/dark photoperiod. For both the PGR and control treatment, 5 repetitions of 1 plant per culture vessel were proliferated. At day 28 following the movement of plants to a treatment medium containing only MS salts and vitamins, plants were subcultured and the number of plants arising from the meristematic base including the original sock plant, were counted.

2.2.3 Callus initiation, maintenance and regeneration

2.2.3.1 Broad scale assessment of callus initiation from seedling derived explants of numerous carrot cultivars

As an initial indicator of cultivar and explant viability, hypocotyl, basal cotyledon and root explants derived from 3 week old seedlings (Figure 2.1) were assessed for their ability to produce callus on agar solidified MS medium containing 0.5 µM 2,4-D.

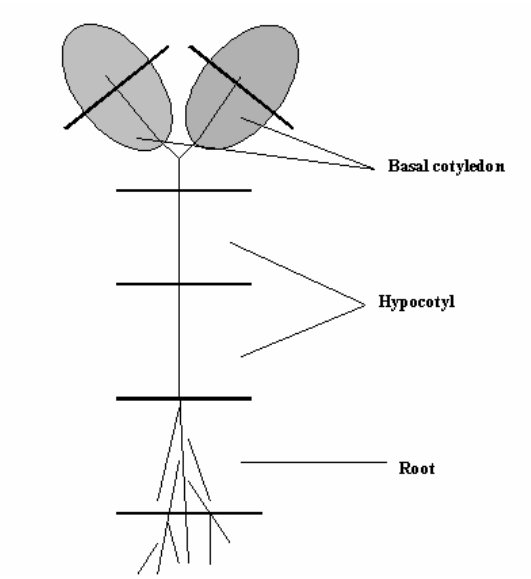

Figure 2.1. Location of carrot seedling derived explants

Fifty of each of the three explant types, per cultivar were assessed on treatment medium. Callus assessment, at day 28 following the subculture of seedling-derived explants to PGR-containing medium, was based on observations of callus produced from explants for each PGR variation. Individual explants were not assessed at this stage. A score was assigned for each PGR variation based on callus morphology using a rating system (Figure 2.2). Nodular or friable callus produced at one or both ends of seedling explants, which was creamy/white in colour, was considered to have embryogenic potential and was assigned a high score. Smooth and watery callus produced in small quantities was not considered to possess embryogenic potential and was assigned a low score.

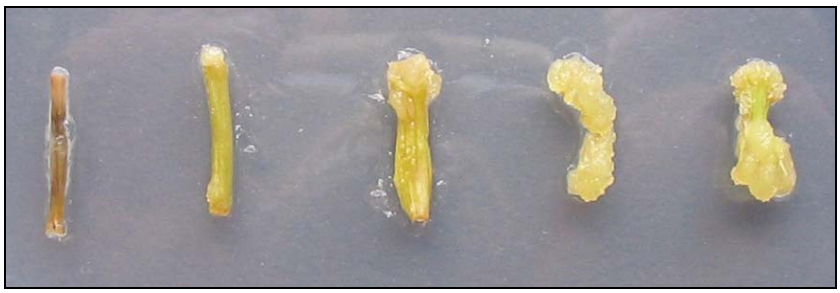

Figure 2.2. Indicative carrot callus morphology for rating system to determine the Callus Quality Score. 0 = explant dead; 1 = explant alive, no callus; 2 = callus at ends of explant; callus typically yellow and not friable; 3 = small amount of callus along length of explant; callus watery looking and not friable; 4 = callus along length of explant with more cells proliferation at ends; callus becoming friable; 5 = callus in large knobs at ends of explants; callus white and friable.

2.2.3.2 Callus initiation from petiole explants

Four-week-old petioles of carrot cultivars Crusader and Stefano were excised into sections of 10 mm and used in callus initiation experiments. Callus was induced on petioles grown on agar solidified MS medium supplemented with 0, 0.5, 1.0 and 5.0 µM 2,4-D and supplemented with timentin (150 µg/mL) to prevent bacterial contamination. For the three treatments comprising 0, 0.5 and 1.0 µM 2,4-D ten

repetitions of 10 explants was conducted and for the remaining treatment containing 5.0 µM 2,4-D five repetitions of 10 explants each was carried out. The explants were maintained at 25ºC in the dark for a period of four weeks.

At day 28 following movement of petiole explants to treatment medium, the number of explants producing callus as a percentage of total explants was calculated for each repetition within each treatment. A score of callus quality was assigned to each callus explant based on callus morphology using the rating system described in Figure 2.2.

2.2.3.3 Callus maintenance

Callus used for maintenance experiments was derived from the optimum callus initiation treatments as determined in section 2.2.3.1. Following a four week callus initiation period, callus was excised from explants and placed on callus maintenance medium (CM) containing agar-solidified MS media containing 0.5 µM 2,4-D and 150 µg/mL of timentin. For callus proliferation, explants were maintained at 25ºC in the dark for a period of eight weeks. At four weeks the callus explants were subcultured to fresh growth medium.

Following the four-week callus proliferation period callus explants were weighed and a comparison made between treatments. Due to the destructive nature of weighing callus explants and the requirement to continue with callus regeneration experiments, only 40 callus explants per treatment were weighed for each cultivar.

2.2.3.4 Callus regeneration

To determine the optimal tissue culture medium to regenerate plantlets from callus via somatic embryos callus explants were moved to agar solidified MS medium without PGR or containing 0.5 µM zeatin. A control regeneration medium consisting of solidified MS containing 0.5 µM 2,4-D was included. All callus regeneration media are supplemented with timentin (150 mg/L) to prevent bacterial contamination. For callus regeneration, explants were maintained at 25ºC with a 16h/8h light/dark photoperiod. For each of the three callus regeneration media, 5 repetitions of 10 explants each were carried out. At day 14 following the movement of callus to callus regeneration media, preliminary observations were made on callus morphology and colour change. At day 35 (week 5) the number of callus explants regenerating as a percentage of total callus explants was determined. Positive regeneration was defined as somatic embryos producing shoots with defined cotyledons and attached roots indicative of seedling germination. Observations of cell proliferation and callus growth were also recorded.

2.2.4 Analysis

Callus quality score and % production was analysed using at least five biological replications. The General Linear Model (GLM) function, within MINITAB for Windows Version 14 (Minitab® Inc, 2003), was used to determine the occurrence of significant differences between treatment means ($P<0.05$) for media, explant and cultivar variables for the purpose of determining optimal media constituents for somatic embryogenesis from carrot tissue. Different letters were assigned to

treatment variables to indicate those treatments that differed significantly from each other following comparison of significant differences.

2.3 Results

2.3.1 Seed sterilisation

The seed sterilisation protocol prevented fungal contamination from carrot seed of both cultivars Stephano and Crusader while maintaining satisfactory seed viability (Table 2.1).

Table 2.1 Effect of the sterilisation protocol on carrot seed viability and pathogen contamination at +7 days post treatment

Cultivar	Germination (%)*	Contamination (%)+	
		Fungal	Bacterial
Stefano	73	0	83
Crusader	84	0	34

* Germination calculated as a percentage of total seeds incubated, excluding those contaminated. + Contamination calculated as a percentage of total seeds incubated.

Bacterial contamination however, was not prevented in neither Stefano nor Crusader seed (Table 2.1). Bacterial contamination only became evident following the emergence of the radical from the seed coat and remained localised around the germinating seed. Subsequent seed germination of both cultivars resulted in similar infection levels indicating an endogenous source of contamination. The addition of 150mg/L Timentin to the germination medium successfully controlled the proliferation of endogenous bacteria and this treatment was used as a bacterial control measure in all carrot regeneration and micropropagation experiments conducted in this study.

2.3.2 Micropropagation

The use of cytokinin-enhanced plant growth medium containing 5 µM BAP had a significant effect on meristematic proliferation from both Stefano and Crusader stock plants following 28 days on treatment medium (Table 2.2). For Stefano, the treatment medium produced close to 6 times as many plants as the control medium, while for Crusader the number of plants produced on treatment medium was nearly 8 times that of control medium.

Table 2.2. Effect of 5 µM BAP on meristematic proliferation from sub-cultured stock plants of carrot cultivars Stefano and Crusader.

	Number of plants arising from original stock plant*											
	Stefano						Crusader					
BAP conc. (µM)	Repetitions						Repetitions					
	1	2	3	4	5	Total	1	2	3	4	5	Total
0	1	1	1	1	1	5	1	1	1	1	1	5
5	5	3	7	4	9	28	15	8	4	5	7	39

*Number includes original stock plant.

The presence of BAP in the treatment medium affected growth form. Figure 2.2a shows cultivar Crusader 28 days post subculture on control medium without BAP (right) compared to treatment medium containing 5 µM BAP (left). Different growth habits were also seen between cultivars grown on control medium, with Crusader growing more prolifically than Stefano, which tended to produce shorter petioles that were darker and thinner than those of Crusader (Figure 2.2b.). One common occurrence between cultivars on both control and treatment medium was the production of callus at the base of plantlets (Figure 2.2b.). Basal callusing prohibited the emergence of roots directly from the meristematic base.

Figure 2.2. a. Effect of 5 µM BAP on stock plant proliferation of carrot cultivar Crusader. Left: Plantlet grown on control medium (no BAP) showing no signs of proliferation. Right: Plantlet grown on treatment medium containing 5 µM BAP showing proliferation of new plantlets from meristematic base. b. Growth habit and basal callusing of Crusader (left) and Stefano (right) stock plants grown on control medium.

2.3.3 Callus initiation

2.3.3.1 Callus initiation from seedling derived explants of five carrot cultivars

Comparison of the quality and quantity of callus production from seedling-derived explants of carrot cultivars Crusader, Kendo, Murdoch, Omeros and Stefano showed similar trends between explant type (Table 2.3). One noticeable variation was the cultivar Kendo which had a low percentage callus production from both root and hypocotyl explants in comparison to the other carrot cultivars. Callus production was generally greater from hypocotyl and cotyledon explants than from root explants. Callus initiated from root explants was smooth and watery, and therefore not considered to possess embryogenic potential. Although hypocotyl and cotyledon explants showed a similar capacity to generate callus, the uniformly nodular and friable callus generated from hypocotyl tissue potentially has a greater capacity for somatic embryogenesis than callus initiated from cotyledon explants. Results of this experiment indicate that carrot cultivars Crusader, Murdoch, Omeros and Stefano are amenable to the application of 2,4-D for the production of callus from hypocotyl explants.

Table 2.3. Description of the effect on callus initiation from seedling derived explants of carrot cultivars Crusader, Kendo, Murdoch, Omeros and Stefano following a 28-day incubation on initiation medium containing 0.5 µM 2,4-D.

	Explant type		
Cultivar	Root	Hypocotyl	Cotyledon
Crusader	80% of explants produced callus. Callus creamy yellow, produced smoothly along entire root.	100% of explants produced callus. Callus creamy yellow, nodular and friable at ends, smooth in middle.	100% of explants produced callus. Callus beginning on basal end, some callus white and dry looking.
Kendo	30% of explants produced callus. Callus creamy yellow, smooth blobs joining to form continuous line of callus along root piece.	50% of explants produced callus. Some callus browning, not nodular and not covering entire explant.	100% of explants produced callus. Callus beginning on basal ends; callus browning, smooth and globular.
Murdoch	100% of explants produced callus. Callus creamy yellow, variable production, mostly in small smooth blobs.	100% of explants produced callus. Callus creamy yellow with big clumps of friable callus at one or both ends.	98% of explants produced callus. Callus beginning at basal ends, some callus white and dry.
Omeros	100% of explants produced callus. Callus creamy yellow, smooth and watery, covering entire explant.	100% of explants produced callus. Callus creamy yellow, nodular at ends, covering entire explant.	100% of explants produced callus. Callus beginning on basal ends, yellow and smooth.
Stefano	70% of explants produced callus. Callus form variable, mostly small smooth creamy/yellow to yellow/brown blobs.	75% of explants produced callus. Callus friable creamy yellow clumps at explant ends.	100% of explants produced callus. Smooth callus located at basal ends.

2.3.3.2 Callus initiation from petiole explants

The effect of 2,4-D concentration on viable callus initiation from petiole explants varies significantly for both carrot cultivars. The mean callus quality score varied significantly between the different 2,4-D concentrations for both Stefano and Crusader, with petiole explants grown on initiation medium containing 0.5 µM 2,4-D producing the highest callus mean quality score of 3.9 and 4.4 respectively. These scores differed significantly for both cultivars from the mean callus quality scores of callus generated from all other callus initiation treatments.

Table 2.4. Effects of 2,4-D on callus quality and production from petiole explants of carrot cultivars Stefano and Crusader

	Callus quality score*		% callus production +	
	Stefano	Crusader	Stefano	Crusader
2,4-D conc. (µM)				
0	0.5 ± 0.06a	0.5 ± 0.06a	3.3 ± 0.3a	4.0 ± 0.4a
0.5	3.9 ± 0.09b	4.4 ± 0.07e	100 ± 10.0b	100 ± 10.0b
1.0	3.4 ± 0.06c	3.4 ± 0.10c	100 ± 10.0b	94 ± 9.0b
5.0	2.2 ± 0.11d	2.9 ± 0.05f	78 ± 11.0c	100 ± 14.0b

*Values represented are the mean callus quality score ± s.e. For each treatment (n=100 for 0, 0.5 and 1.0 µM 2,4-D, n=50 for 5 µM 2,4-D). * Callus Quality Score. 0 = explant dead; 1 = explant alive, no callus; 2 = callus at ends of explant; callus typically yellow and not friable; 3 = small amount of callus along length of explant; callus watery looking and not friable; 4 = callus along length of explant with more cells proliferation at ends; callus becoming friable; 5 = callus in large knobs at ends of explants; callus white and friable. + Callus production as a percentage of total petiole explants treated. For comparison of treatment means for each callus response, significant differences are indicated with different letters ($P<0.05$).

Treatments containing 0.5 µM 2,4-D induced high percentages of callus production (Table 2.4). Percentage callus production showed high similarity between treatments and cultivars. Percentage callus production from the control treatment containing no 2,4-D was significantly lower than all 2,4-D treatments for both cultivars. PGR treatment of 5 µM 2,4-D produced significantly lower percentage of callus from petiole explants compared to other 2,4-D treatments on the cultivar Stefano.

2.3.4 Callus maintenance

Callus generated from petioles placed on medium containing 0.5 and 1.0 µM 2,4-D that obtained a mean callus quality score of 3.5 or greater, was used for callus proliferation. Assessment of callus weight following the 28 days of callus proliferation indicated that callus generated from petioles treated on medium containing 0.5 µM 2,4-D weighed significantly more than callus generated from petioles treated on medium containing 1.0 µM 2,4-D (Table 2.5). Callus generated from Stefano and Crusader petioles on 0.5 µM 2,4-D had a mean weight of 0.235 g and 0.225 g respectively following the 28 day proliferation period. There were no significant differences between cultivars, with both Stefano and Crusader responding similarly to both treatments. In support of the results presented in Table 2.5, visual assessment of callus morphology four weeks following the movement of callus to callus maintenance medium, indicated callus generated from petioles treated on medium containing 0.5 µM 2,4-D was more friable and nodular than callus generated on medium containing 1.0 µM 2,4-D.

Table 2.5. Effect of 2,4-D on callus weight (following a 4 week callus proliferation stage) from carrot cultivars Stefano and Crusader.

	Callus weight (g)*	
	Stefano	Crusader
2,4-D conc. (µM)		
0.5	0.235 ± 0.009a	0.225 ± 0.014a
1.0	0.188 ± 0.008b	0.186 ± 0.009b

*Values represented are treatment mean weight (g) ± s.e. (n=73 & 41 for Stefano and Crusader respectively at 0.5 µM 2,4-D; n=67 & 41 for Stefano and Crusader respectively at 1.0 µM 2,4-D). For comparison of treatment means for each callus response, significant differences are indicated with different letters ($P<0.05$).

2.3.5 Callus regeneration

At day 14 following the movement of callus to callus regeneration medium containing different plant growth regulators, preliminary observations were made on callus morphology and colour change (Table 2.6). At this early stage of regeneration, callus was beginning to show signs of change in both morphology and colour. Callus from both cultivars on regeneration medium with no PGR and regeneration medium containing 0.5 µM zeatin, had begun to change colour from creamy yellow to green with minimal purple regions (Figure 2.4a). Callus from both cultivars on control medium containing 0.5 µM 2,4-D showed a predominant colour change to purple and was beginning to develop highly embryogenic callus forms, with individual embryos at varying developmental stages that were visible under magnification (Figure 2.4b). Stefano and Crusader callus undergoing callus regeneration treatment of 0.5 µM zeatin appeared to possess higher embryogenic potential than both treatment (no PGR) and control (0.5 µM 2,4-D) media.

Figure 2.4. Regeneration of callus fourteen days following the movement of explants to treatment medium. a. Stefano callus explant on medium containing 0.5 µM zeatin. b. Magnification of heart-shaped embryo generated from Crusader callus on treatment medium without PGR.

Table 2.6. Observations on the effect of PGR treatment after 14 days incubation on the regeneration of plantlets from Stefano and Crusader callus tissue.

	Observations
	Stefano
PGR	
-MS	Colour change to green; 75% of explants with white embryogenic callus growth; some shoots emerging.
0.5 µM zeatin	Colour change to green and purple; 25% of explants with white embryogenic callus growth; this callus showing high % of embryo to shoot formation.
0.5uM 2,4-D	Colour change to purple; 25% of explants with white callus growth.
	Crusader
-MS	Change of to green and purple; may have beginnings of embryogenic forms
0.5 µM zeatin	Colour change to green with minimal purple; 50% of explants with white embryogenic callus growth; some shoots emerging.
0.5uM 2,4-D	Colour change (slight) green and purple; 40% of explants with white embryogenic callus growth.

At day 35 the number of callus explants regenerating and/or proliferating as a percentage of total callus explants was determined (Table 2.7).

Table 2.7. Effect of PGR treatment on regeneration of viable plantlets from callus of carrot cultivars Stefano and Crusader after 35 days incubation.

	Callus regenerating (%) *		**Callus proliferation (%) ***	
	Stefano	Crusader	Stefano	Crusader
PGR				
0.5 µM 2,4-D $^{+}$	00.0± 0.0a	00.0 ± 0.00a	00.0 ± 0.0a	00.0 ± 0.0a
No PGR	64.0 ± 18.6b	70.0 ± 7.0c	56.0 ± 16.3bd	88.0 ± 5.8c
0.5 µM zeatin	76.0 ± 9.3c	70.0 ± 7.0c	82.0 ± 8.6c	70.0 ± 7.7cd

*Callus regeneration and proliferation as a percentage of total callus explants treated. Values represented are treatment means ± s.e. (n=50 for Stefano and Crusader respectively at 0.5 µM 2,4-D and 0.5 µM zeatin and Stefano at no PGR; n=92 for Crusader at no PGR). For comparison of treatment means for each callus response, significant differences are indicated with different letters ($P<0.05$). $^{+}$Control treatment.

Following a 35 day callus proliferation period, explants of cultivars Stefano and Crusader that were treated on control medium containing 0.5 µM 2,4-D showed no signs of regeneration (ie. no production of viable plantlets) or callus proliferation (Table 2.7). The results from the remaining two treatments varied significantly between the two carrot cultivars. For Stefano, callus explants regenerating on medium containing 0.5 µM zeatin showed a significantly higher percentage regeneration than the "No PGR" treatment and control medium containing 0.5 µM 2,4-D, with 76% of explants producing viable plantlets with fully formed cotyledons and attached roots. Percentage callus proliferation was also significantly higher for Stefano explants on this treatment medium, with 86% of callus explants showing some degree of growth. For Crusader, callus explants regenerating on medium containing 0.5 µM zeatin and no PGR showed significantly higher percentage regeneration than the control medium. However, treatments did not vary significantly from each other, with both treatments promoting viable plantlets with fully formed

cotyledons and attached roots from 70% of callus explants. Likewise, callus proliferation of Crusader explants was not significantly different between treatments. Comparison between cultivars indicated Stefano explants performed better on treatment medium containing 0.5 µM zeatin, while Crusader explants performed better on treatment medium without PGR (Table 2.7). Although the results show that medium supplemented with 0.5 µM zeatin was most successful for regeneration from callus explants of cultivar Stefano, plantlets emerging from callus treated on this medium were vitrified and malformed for both cultivars. It was concluded therefore, that the treatment medium without PGR was best able to generate well-formed, viable plantlets from undifferentiated callus of both Stefano and Crusader.

The progression of callus from an undifferentiated state through somatic embryogenesis resulting in the 'germination' of viable plantlets (or 'seedlings') was monitored on MS medium containing no PGR (Figure 2.5). Fully formed seedlings were most likely to regenerate from callus that was creamy yellow to green in colour (Figure 2.5a) that resulted in significant proliferation (Figure 2.5bc). Yellow callus did not give rise to 'germinated' embryos. Although white callus appeared highly embryogenic, it gave rise to malformed plantlets without pigmentation. There was a positive correlation between increased callus size (callus proliferation) and regeneration, with larger callus explants possessing greater capacity for regeneration.

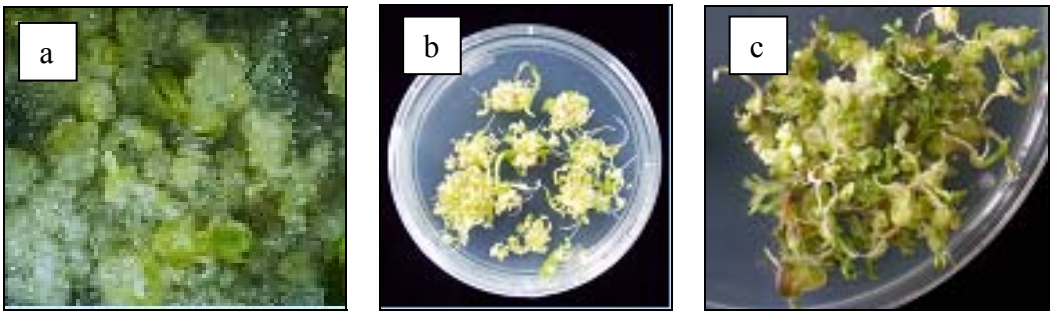

Figure 2.5 Effect of removing PGR from MS medium on callus cultures of carrot cultivar Crusader. a. Magnification of regenerating callus at early stage, with green cotyledons evident. b. Regeneration from friable embryogenic callus. c. Regeneration more advanced, with significant cell proliferation and 'germination' of fully formed 'seedlings'.

2.4 Discussion

Many reports exist of seedling-derived explants, primarily hypocotyl explants, being amenable to PGR treatment for callus production (Smith and Street 1974; Kamada and Harada 1978; Kitto and Janick 1985; Takaichi and Oeda 2000; Jiménez and Bangert 2001; Chen and Punja 2002). Results reported in Section 2.3.3.1 indicated that seedling-derived hypocotyl explants of carrot cultivars Stefano and Crusader were responsive to the manipulation of exogenous PGR treatment for the production of viable callus. The use of seedling-derived explants for tissue culture and transformation experiments would require continual sterilisation and germination of seed.

Our results indicate that carrot petioles derived from stock plant cultures are equally responsive to PGR manipulation for the production of viable callus. The inclusion of

BAP in plant proliferation medium greatly enhances the ability of subcultured Stefano and Crusader stock plants to multiply from their meristematic base. This finding is consistent with those reported by Drew (1979). The use of petiole explants derived from stock plant cultures of cultivars Stefano and Crusader maintained *in vitro* avoids the requirement for fresh seedling material, allowing for a continual supply of sterile plant material for tissue culture and transformation experiments.

The morphological characteristics of Stefano and Crusader callus obtained when petioles were cultured on medium containing varying concentrations of 2,4-D, were similar to carrot embryogenic and non-embryogenic callus described elsewhere (Smith and Street 1974; Sasaki *et al*. 1994). As described by George *et al*. (1993), both embryogenic and non-embryogenic callus commonly arose from the same callus explant. We showed a PGR treatment of 0.5 µM 2,4-D to be optimal for the initiation of embryogenic callus from petiole explants of both carrot cultivars. These findings are consistent with those of Hartmann *et al.* (1997) who determined that auxin at a moderate to high concentration is necessary to promote callus production. The effects of exogenously applied 2,4-D on the induction of embryogenic callus are well documented (Dudits *et al*. 1991; Yeung 1995). The majority of reports for carrot regeneration have indicated the use of MS nutrient medium supplemented with 2,4-D at 5µM for callus initiation from hypocotyl and root explants (Kamada and Harada 1978; Kitto and Janick 1985; Jiménez and Bangert 2001; Chen and Punja 2002). In this chapter both cultivars responded best to the application of 0.5 µM 2,4-D for the generation of embryogenic callus from petiole explants and these results are consistent with those reported by Drew (1979). Cultivars Crusader and Stefano were used as a source of explant material for subsequent callus initiation and regeneration experiments. This decision was based on their close relatedness to Nantes type carrots, the carrot type most commonly grown for commercial carrot production in Australia.

The requirement of plants for differing concentrations of auxins for callus proliferation and embryo development can sometimes be variety dependent (Vasil 1984). In this instance both Stefano and Crusader responded similarly to the same PGR treatment and this could be a reflection of their both being Nantes type carrots. The addition of 0.5 µM 2,4-D in callus initiation medium was implemented for subsequent regeneration and transformation experiments using stock plant-derived petiole explants.

It is widely reported that the removal of all plant growth regulators will stimulate the germination of somatic embryos from carrot cell cultures (Smith and Street 1974; Drew 1979; Kitto and Janick 1985; Roustan *et al.* 1989; Nissen 1994; Jiménez and Bangert 2001; Takaichi and Oeda 2000; Chen and Punja 2002). The inclusion of 0.5 µM zeatin in callus regeneration medium has also been shown to effectively initiate somatic embryogenesis in carrot cell cultures (Fujimura and Komamine 1975, 1979; Fujimura *et al* 1980). Work reported in this chapter shows that somatic embryogenesis from carrot callus cultures can be initiated by moving callus cultures from auxin-containing medium to auxin-free medium or medium containing 0.5 µM zeatin. Although a higher percentage of callus that was moved to medium containing 0.5 µM zeatin underwent somatic embryogenesis, only those plantlets regenerated from callus regenerated on auxin-free medium were well formed and not vitrified.

The regeneration system described here is useful for the differentiation of single or small clusters of cells to whole plants. Somatic embryogenesis systems that regenerate whole plants from single cells at high frequencies, opens many possibilities in plant breeding including transformation systems that will enable the generation of transgenic plants (Osuga and Komamine 1994). The optimisation of somatic embryogenesis-based tissue culture systems for carrot cultivars reported in this chapter will enable the production of transgenic carrot lines. Carrot petiole explants will be inoculated with *Agrobacterium tumefaciens* carrying reporter genes and antiviral ihpRNA cassettes and transformed cells subsequently regenerated via somatic embryos.

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3.0 Tissue culture and regeneration of *Apium graveolens* var.*dulce* cultivars Summit and Tendercrisp

3.1 Introduction

Celery (*Apium graveolens* var.*dulce*), like carrot, is highly embryogenic. High frequency embryogenic regeneration from callus cultures is an ideal method to rapidly multiply larger genetically identical plant populations (Browers and Orton 1986). Since the first report of *in vitro* culture and adventitious embryos in celery (Reinert *et al.* 1996), improved methods have been developed for culture initiation, maintenance and regeneration. Callus can be initiated from celery by culturing explants on plant growth medium containing a combination of plant growth regulators such as 2,4-D together with BAP (Wright and Lacy 1988; Nadel *et al*. 1989) or kinetin (Chen 1976; Williams and Collin 1976a; Zee and Wu 1979; Nadel *et al*. 1990a; Saranga and Janick 1991; Onishi *et al.* 1992; Evanor *et al*. 1994). The removal of both auxin and cytokinin simultaneously, or the removal of auxin and cytokinin sequentially will initiate regeneration. In this chapter, the effects of varying concentrations of 2,4-D in combination with BAP or kinetin at different concentrations on callus initiation from seedling- and petiole-derived tissue for optimal callus proliferation of celery cultivars Summit and Tendercrisp were explored. The effects of removing auxin or the addition of cytokinin to plant growth medium on the regeneration capacity from callus explants was also investigated.

The proliferation of seedling-derived meristematic shoot tips for the production of stock tissue culture plantlets is described. To date, no reports exist for the clonal propagation of celery plants from meristematic material. Meristematic proliferation provides a method of clonal multiplication in a relatively short time and can be useful for the maintenance of transgenic or non-transgenic clonal lines. Meristematic proliferation also avoids the requirement of plant cells to revert to an undifferentiated state as is indicative of somatic embryogenesis. However it is possible that a well-developed somatic embryogenesis system for celery proliferation provides a better avenue for the production of large numbers of clonal plants, rendering meristematic proliferation obsolete.

To determine which plant growth regulator (PGR) treatments may be successfully applied for the micropropagation of celery plants, the system for clonal propagation of strawberry plants (*Fragaria ananassa*) was assessed. Strawberries have a similar growth physiology to that of celery, with the meristematic material found at the base of plants and at the juncture of petioles and roots. The cytokinin treatment most commonly used for micropropagation of strawberry plants was tested for its ability to multiply celery plants from meristematic material. Three alternative cytokinin analogues were also assessed for their ability to initiate the production of plantlets from meristematic material.

3.2 Materials and methods

3.2.1 Plant materials

Celery (*Apium graveolens* var. *dulce*) seeds of cv. Summit (Henderson Seeds) and Tendercrisp (South Pacific Seeds Ltd) were surface sterilised in 70% ethanol containing 0.01% Tween 20 for 1 min and in 2% available chlorine and 0.01% Tween 20 for 20 min with shaking. Seeds were washed three times in sterile deionised water, then germinated and maintained in sterile tubs for three weeks on agar-solidified, hormone-free MS medium (Murashige & Skoog 1962) at 25ºC with a 16h/8h light/dark photoperiod. For the assessment of seed viability and control of microbial contaminants to ensure adequate seedling plant material for initial callus initiation experiments, a germination trial was conducted and consisted of 5 repetitions of 20 seeds each. The non-contaminated germinated seeds were calculated as a percentage of the number of seeds germinated at day 21 following placement of seed on germination medium.

Following germination, seedlings were either used as a source of explant material for callus initiation experiments or as a source of meristematic shoot tips for stock plant culture. Stock plants were maintained on hormone-free MS medium, routinely subcultured every 8 weeks and used as a source of explant material for subsequent callus initiation experiments. Subcultures of stock plants involved the removal of roots and callus material from below the meristematic base and the removal of petiole and leaf material to a height of approximately two cm from the meristematic base, leaving 2-3 young, actively growing leaves.

3.2.2 Micropropagation

To assess the effect of cytokinin on the proliferation of Summit and Tendercrisp plantlets from meristems for the purpose of generating stock plants, meristematic shoot tips were placed on MS medium supplemented with 5 µM of 6-benzylaminopurine (BAP), thiadurzine (TDZ), zeatin or kinetin. Plants were maintained at 25ºC with a 16h/8h light/dark photoperiod. For all cytokinin treatments, 10 repetitions of 5 shoot tips each were tested. At day 35 (week 7), following the movement of plants to MS medium, plants were visually assessed based on the generation of large plants with normal form and shape.

3.2.3 Callus initiation, maintenance and regeneration

3.2.3.1 Broad scale assessment of callus initiation from seedling derived explants of celery cultivars Summit and Tendercrisp

As an initial indicator of explant viability, root hypocotyl, apical and basal cotyledon explants derived from 3 week old Summit seedlings (Figure 3.1) were assessed for their ability to produce callus on treatment media consisting of agar solidified MS medium containing 0.25, 0.5 or 2.5, 5 or 10 µM 2,4-D alone or in combination with 2.5 µM kinetin. Agar solidified MS medium without 2,4-D was used as control for comparison with treatment medium.

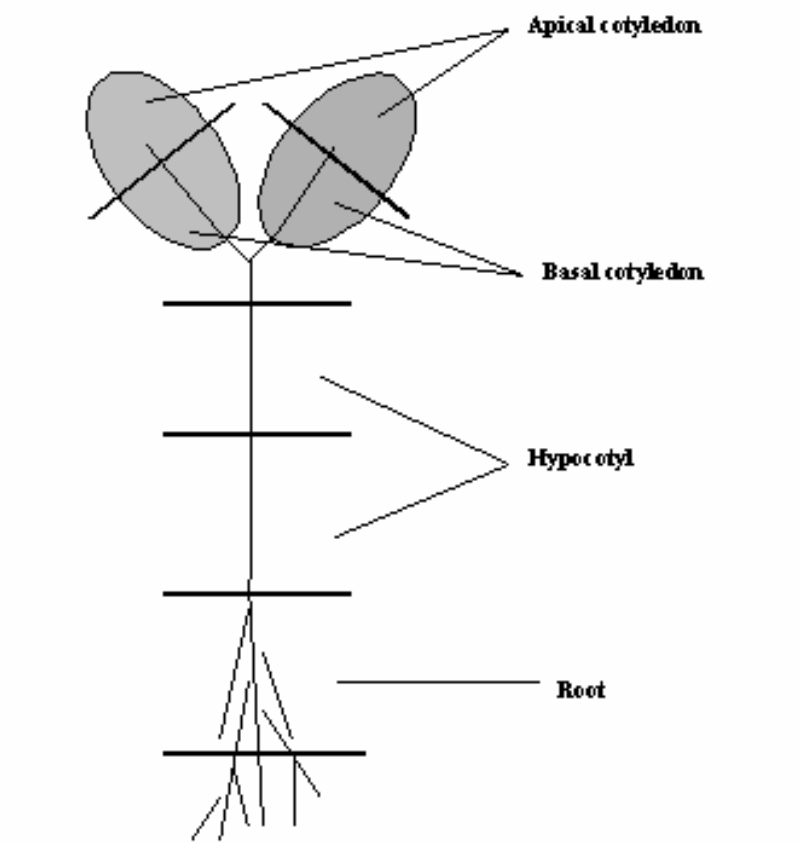

Figure 3.1. Source for seedling derived explants

For each of the four explant types and 6 treatment media, 5 repetitions of 10 explants each were assessed on treatment medium. Callus assessment at day 28 following the subculture of seedling-derived explants to treatment media was based on the observations of callus produced from each explant. A callus quality score was assigned to each callus explant based on callus morphology using the rating system depicted in Figure 3.2. Nodular or friable callus, which was creamy/white in colour, was considered as having embryogenic potential and was assigned a high score (Fig 3.2). Smooth and watery callus was considered not to possess embryogenic potential and was assigned a low score (Fig 3.2).

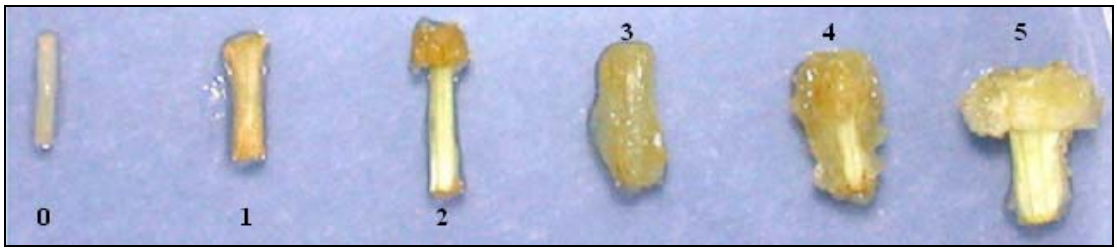

Figure 3.2. Indicative callus morphology of celery for rating system. 0 = explant dead;1 = explant alive, no callus; 2 = callus at ends of explant; callus typically yellow and not friable; 3 = small amount of callus along length of explant; callus watery looking and not friable; 4 = callus along length of explant with more cells proliferation at ends; callus becoming friable; 5 = callus in large knobs at ends of explants; callus white and friable.

To further examine the effect of PGR treatment on callus initiation from seedling explants, Summit and Tendercrisp hypocotyl and basal cotyledon explants were incubated on MS medium containing 0.25 μM 2,4-D in combination with 2.5 μM kinetin, 5.0 μM kinetin, 0.5 μM BAP or 5.0 μM BAP. MS media containing 0. 25 μM 2,4-D without the inclusion of cytokinin was used as a control. At day 28 following movement of seedling-derived explants to the treatment medium (MS medium containing 0.25 μM 2,4-D), the number of explants producing callus as a percentage of total explants was calculated for each repetition within the treatments. At this stage, callus with a rating of 3.5 or more was removed from explants and moved to callus maintenance medium.

3.2.3.2 Callus initiation from petiole explants

Four-week-old petioles of celery cultivars Summit and Tendercrisp were excised into 10 mm sections and used in callus initiation experiments. Callus induction from petioles was attempted on agar solidified MS medium supplemented with 0.25, 0.5, 2.5, 5.0 or 25 μM 2,4-D in combination with 0.5, 2.5 or 5.0 μM kinetin or 0.5 or 5.0 μM BAP. For comparison with auxin/cytokinin-containing callus initiation medium, petioles were also incubated on media containing 0, 0.25, 0.5, 2.5, 5.0 or 25 μM 2,4-D. For each of the 25 auxin/cytokinin combinations, and each of 6 controls, 5 repetitions of 10 explants each were carried out for each celery cultivar.

The induction of callus from petiole explants was also attempted on agar solidified B5 medium (Gamborg *et al*. 1968) containing 100mg/L serine, 2.5 μM 2,4-D and 3.0 μM kinetin. For comparison with auxin/cytokinin-containing B5 medium, callus was also induced on PGR-free B5 salts and vitamins in combination with 100mg/L serine and MS medium in combination with 2.5 μM 2,4-D and 3.0 μM kinetin. For each celery cultivar, 5 repetitions of 10 explants each were carried out.

At day 35 following movement of petiole explants to callus initiation media, the number of explants producing callus as a percentage of total explants was calculated for each callus initiation medium. A score was assigned to each callus explant based on callus morphology using the rating system defined in Section 3.2.3.1. At this stage, callus with a rating of 3.5 or more was considered to possess embryogenic potential and was removed from petiole explants and transferred to callus maintenance medium.

3.2.3.3 Callus maintenance

Celery callus tissue for maintenance and proliferation was derived from the optimum callus initiation treatments as determined in section 3.2.3.2. Following a 35 day callus initiation period, callus was removed from explants and placed on callus maintenance medium (CM), consisting of agar-solidified MS medium containing 0.5 μM 2,4-D. For callus proliferation, explants were maintained at 25ºC in the dark for a period of eight weeks. At four weeks the callus explants were subcultured to fresh CM.

3.2.3.4 Callus regeneration

To determine the optimal medium to regenerate plantlets from celery callus via somatic embryos, explants derived from the optimal callus initiation treatments (both seedling-derived and petiole explants) were moved to agar solidified MS callus regeneration treatment medium without PGR or containing 1.0 µM kinetin. A control callus regeneration medium consisting of agar solidified MS containing 0.5 µM 2,4-D was included. For each treatment medium and celery cultivar, a minimum of 2 repetitions of 10 explants each was tested and explants were maintained at 25ºC with a 16h/8h light/dark photoperiod. At day 7 following the movement of callus to the various callus regeneration treatment media preliminary observations were made on callus morphology and colour change. At day 35 the number of callus explants regenerating, as a percentage of total callus explants, was determined. Positive regeneration was defined as callus producing shoots with defined cotyledons and attached roots indicative of seedling germination. Observations were also made as to cell proliferation and callus growth.

3.2.4 Analysis

Callus score and % production was analysed using at least five biological replications. The General Linear Model (GLM) function, within MINITAB for Windows Version 14 (Minitab® Inc, 2003), was used to determine the occurrence of significant differences between treatment means ($P<0.05$) for media, explant and cultivar variables for the purpose of determining optimal media constituents for somatic embryogenesis from celery tissue. Different letters were assigned to treatment variables to indicate those treatments that differed significantly from each other following comparison of significant differences.

3.3 Results

3.3.1 Seed sterilisation

The seed sterilisation protocol proved 100% effective in preventing fungal contamination from celery seed of both cultivars (Table 3.1). A germination percentage (seed viability) of 84% was obtained from celery cultivar Tendercrisp, with germinated seed showing no signs of bacterial contamination. Although germinated seed of celery cultivar Summit showed no emergence of either bacterial or fungal pathogens; seed germination was lower than that of Tendercrisp at only 27%. The variation in germination percentage between cultivars could be indicative of seed source and subsequently seed quality or age. Larger numbers of Summit seed were germinated to ensure adequate seedling material for subsequent micropropagation and tissue culture experiments,

Table 3.1. Effect of the sterilisation protocol on celery seed viability and sterility at 21 days post treatment

Cultivar	Germination (%)*	Contamination (%)+	
		Fungal	Bacterial
Summit	27	0	0
Tendercrisp	84	0	20

* Germination calculated as a percentage of total seeds incubated, excluding those contaminated. + Contamination calculated as a percentage of total seeds incubated.

3.3.2 Micropropagation

The use of cytokinin-enhanced plant growth medium had a significant effect on proliferation from meristematic shoot tips of celery cultivars Summit and Tendercrisp following 35 days on treatment medium (Table 3.2; Figure 3.3).

Table 3.2. Effect of 5 µM cytokinin treatments on meristematic shoot tip proliferation of celery cultivars Summit and Tendercrisp.

	Observations	
	Summit	Tendercrisp
Cytokinin (5 µM)		
TDZ	Stunted plants; no leaf formation; no roots	Stunted plants; no leaf formation; no roots; petioles yellow
Zeatin	Tiny plants; short petioles; yellowing leaves; some small roots	Medium plants; well formed leaves; yellowing leaves; some root formation
BAP	Small to medium plants; well formed leaves; no roots	Small plants; well formed leaves; yellowing leaves; no root formation
Kinetin	Large plants; long petioles; well formed leaves; many roots	Large plants; long petioles; well formed leaves; many roots

Figure 3.3 shows the effect of cytokinin treatments on cultivar Summit. Both cultivars responded similarly to the application of TDZ, zeatin, BAP and kinetin (Table 3.2). Shoot tips of both cultivars treated on medium containing 5 µM kinetin produced well formed plants (Figure 3.3). Zeatin and BAP treatments produced stunted plants without roots and the TDZ treated plants were malformed (Figure 3.3). All other cytokinin treatments produced stunted, and in the case of the TDZ treatment, malformed plants. For further meristematic shoot tip proliferation for the production of stock plants of celery cultivars Summit and Tendercrisp, plant growth medium was augmented with 5 µM kinetin.

Figure 3.3. Effect of 5 µM cytokinin treatments on meristematic shoot tip proliferation of celery cultivar Summit. Cytokinin treatments from left to right: TDZ, zeatin, BAP and kinetin.

3.3.3 Callus initiation

3.3.3.1 Callus initiation from seedling explants

Initial attempts at generating callus from seedling explants indicated that callus initiation medium containing 0.25 µM 2,4-D in combination with 2.5 µM kinetin was the most successful medium for initiating callus from all explant types (Table 3.3). Hypocotyl explants responded most successfully to this treatment, producing callus with a higher score than callus generated from other explant types (Table 3.3). The callus initiation medium defined in this experiment as possessing the ability to produce large quantities of friable callus from seedling explants (MS containing 0.25 µM 2,4-D and 2.5 µM kinetin) was used as the basis for subsequent callus initiation experiments from both seedling and petiole explants.

The assessment of celery seedling tissue for viable callus production, when grown on MS media containing 0.25 µM 2,4-D and varying cytokinin analogues and concentrations, indicated the celery cultivar Summit responded significantly better to the presence of PGR in plant growth medium than the celery cultivar Tendercrisp (Figure 3.4; Table 3.4).

Following a 28-day incubation period, Summit cotyledon explants on control medium containing 0.25 µM 2,4-D and no cytokinin generated the highest mean callus quality score of 4.8. Callus generated from this treatment was creamy, white in colour and highly friable, covering the entire explant (Figure 3.5) and more than 90% of the explants treated produced callus (Table 3.4). The mean callus quality score of callus generated from Summit cotyledon explants was not significantly different from mean callus quality scores for hypocotyl explants when incubated on treatment medium containing 0.25 µM 2,4-D supplemented with kinetin or BAP. Although the treatment medium containing 5.0 µM BAP did not produce the highest mean callus quality score, callus generated from hypocotyl explants on this treatment medium

appeared larger than callus from other treatments and highly embryogenic, being pale in colour and highly friable (Figure 3.4).

Table 3.3. Effect of varying concentrations of 2,4-D in combination with 2.5 µM kinetin on callus quality from seedling-derived explants of celery cultivar Summit

	Callus Quality Score			
	Explant type			
	Apical cotyledon	Basal cotyledon	Hypocotyl	Root
2,4-D conc. (µM)				
0	1	1	1	1
0.25	1	1	2	1
0.25 + kinetin[+]	3	3	5	4
2.5	2	2	3	2
2.5 + kinetin	2	2	3	2
5.0	1	1	1	1
5.0 + kinetin	1	1	2	1
25.0	1	1	1	1
25.0 + kinetin	1	1	1	1

[+] Callus quality score based on visual assessment: 0 = explant dead; 1 = explant alive, no callus; 2 = callus at ends of explant; callus typically yellow and not friable; 3 = small amount of callus along length of explant; callus watery looking and not friable; 4 = callus along length of explant with more cells proliferation at ends; callus becoming friable; 5 = callus in large knobs at ends of explants; callus white and friable.

Table 3.4. Effect of 0.25 µM 2,4-D in combination with varying concentrations of cytokinin analogues on callus production (defined by percentage callus production) from seedling-derived explants of celery cultivars Summit and Tendercrisp.

	Callus production (%) *			
	Basal Cotyledon		Hypocotyl	
	Summit	**Tendercrisp**	**Summit**	**Tendercrisp**
0.25 µM 2,4-D + cytokinin (µM)				
+ 0 cytokinin	100 ± 0	95 ± 5	90 ± 10	62 ± 12
+ 2.5 kinetin	100 ± 0	100 ± 0	90 ± 6	86 ± 6
+ 5.0 kinetin	100 ± 0	100 ± 0	83 ± 5	74 ± 13
+ 0.5 BAP	100 ± 0	98 ± 2	92 ± 5	78 ± 2
+ 5.0 BAP	90 ± 0	90 ± 10	100 ± 0	86 ± 4

*Values represented are treatment means ± s.e. Callus production as a percentage of total explants treated.

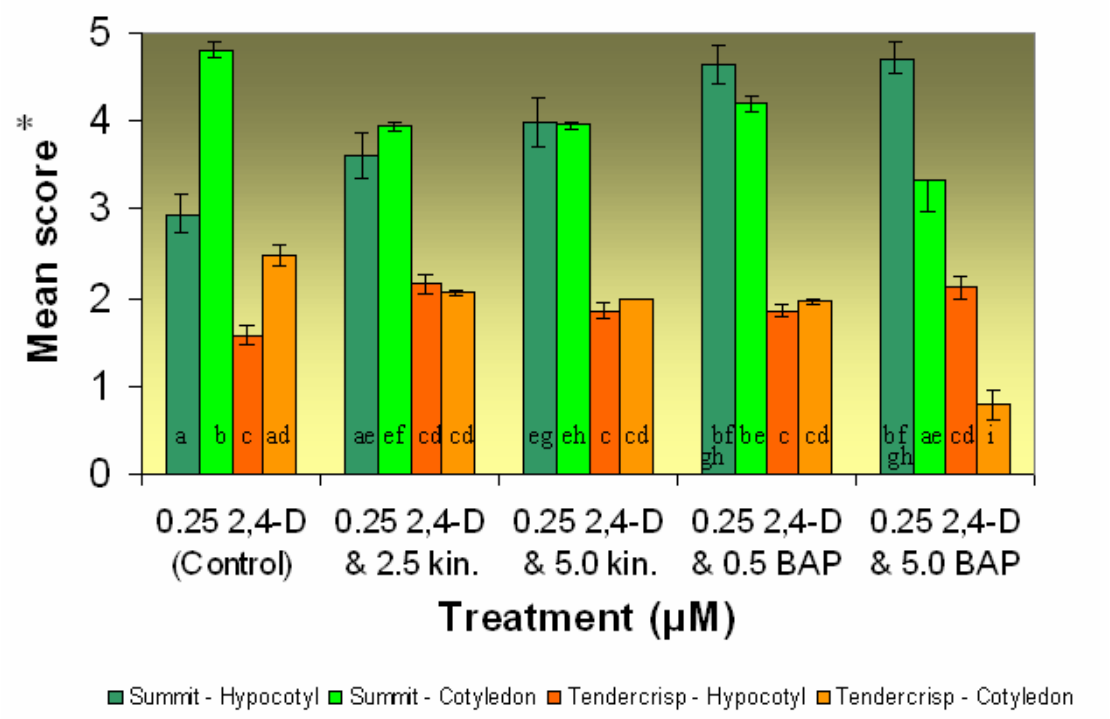

Figure 3.4. Effect of 0.25 µM 2,4-D in combination with varying concentrations of cytokinin analogues on callus production (defined by callus score) from hypocotyl and cotyledon-derived explants of celery cultivars Summit and Tendercrisp.

*Values represented are treatment means ± s.e. For comparison of treatment means for each callus response, significant differences are indicated with different letters ($P<0.05$).

Figure 3.5. Callus production from Summit cotyledon explants grown on callus initiation medium containing 0.25 µM 2,4-D.

The celery cultivar Tendercrisp responded similarly to Summit, with cotyledons incubated on control treatment medium containing 0.25 µM 2,4-D producing the highest callus score following a 28-day incubation period on treatment medium. However the Tendercrisp cotyledon explants produced a mean callus quality score of 2.5 on this treatment medium, which is significantly lower than the highest mean callus quality score of 4.5 for Summit (Figure 3.4). The highest mean callus quality score for Tendercrisp hypocotyl tissue did not differ significantly between treatment media containing any of the four cytokinin combinations tried in this study (Figure 3.4). Callus production from Tendercrisp cotyledon and hypocotyl explants was sub-optimal for all treatments and high variability was seen within treatments.

Unlike mean callus quality scores, which varied greatly between cultivars and treatments, there was little variation between cultivars and treatments for percentage callus production. The most noticeable differences were percentage callus production from Tendercrisp hypocotyl explants, which performed least successfully across all treatments (Table 3.5).

3.3.3.2 Callus initiation from petiole explants

To further explore the capacity for celery plant tissue to produce viable callus, the effects of varying concentrations of 2,4-D, in combination with various cytokinin analogues and concentrations on callus production from petiole explants of cultivar Summit was assessed (Figure 3.6; Table 3.5). Due to the sub-optimal callus production from cultivar Tendercrisp using seedling-derived explants and PGR regimes similar to those applied for the purpose of this experiment (see Section 3.3.3.1), Tendercrisp was excluded from the following experiment.

The effect of 2,4-D concentration in combination with cytokinin analogues on viable callus initiation from petiole explants varied significantly across all treatments. Summit petiole explants grown on medium containing 0.25 µM 2,4-D with either 5.0 µM kinetin or 5.0 µM BAP produced callus with the highest mean quality score of 5. All explants placed on the treatment medium containing 0.25 µM 2,4-D and a cytokinin produced callus including the treatment medium containing 0.25 µM 2,4-D and 5.0 µM BAP (Table 3.5). This treatment medium, for the induction of viable embryogenic callus from Summit petiole explants, was used for subsequent *A. tumefaciens* transformation experiments.

Table 3.5. Effect of different 2,4-D concentrations in combination with varying concentrations of cytokinin analogues on callus production (defined by percentage callus production) from petiole explants of celery cultivar Summit.

	Callus production (%) * **Cytokinin (µM)**					
	0	0.5 kinetin	2.5 kinetin	5.0 kinetin	0.5 BAP	5.0 BAP
2,4-D (µM)						
0	0 ± 0	-	-	-	-	-
0.25	96 ± 2.5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.5	96 ± 2.5	100 ± 0	98 ± 2.0	100 ± 0	100 ± 0	94 ± 2.4
2.5	92 ± 3.7	98 ± 4.5	98 ± 2.0	100 ± 0	100 ± 0	100 ± 0
5.0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	96 ± 2.4	100 ± 0
25	100 ± 0	100 ± 0	10 ± 3.6	22 ± 6.6	8 ± 3.7	11 ± 5.1

*Values represented are treatment means ± s.e. (n = 50). Callus production as a percentage of total explants treated.

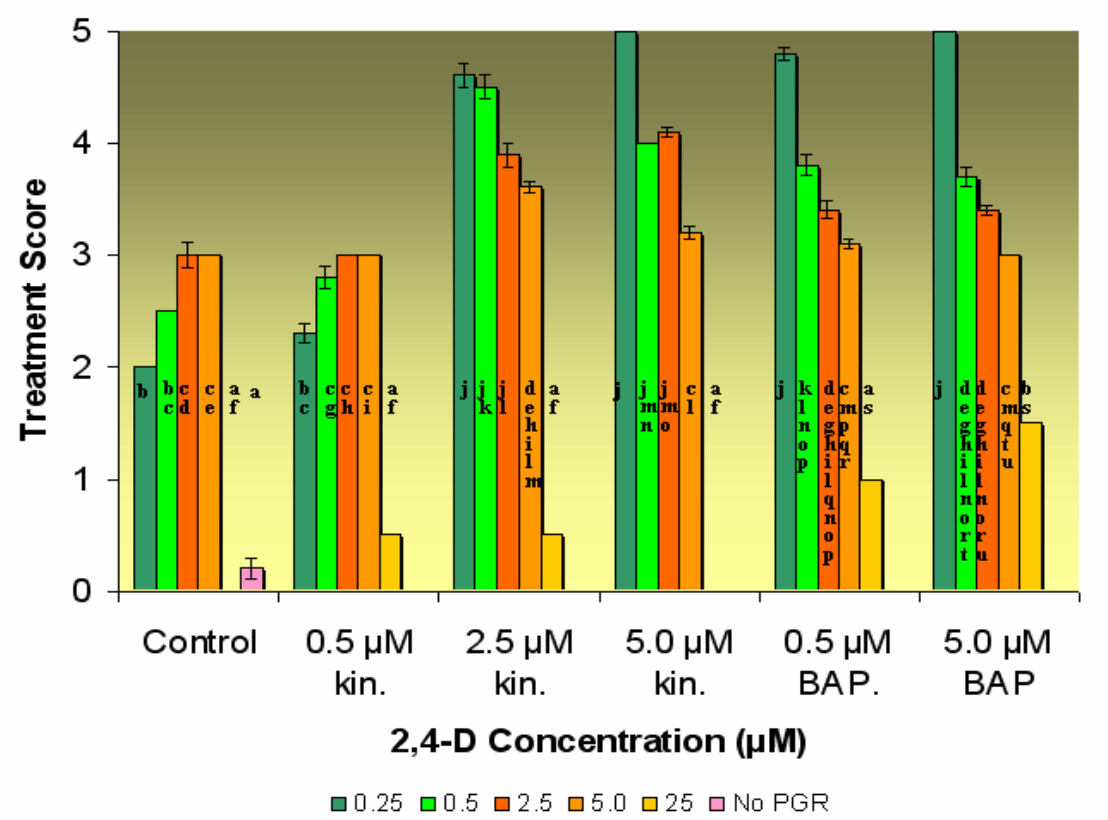

Figure 3.6. Effect of different 2,4-D concentrations in combination with varying concentrations of cytokinin analogues on callus production (defined by callus quality score) from petiole explants of celery cultivar Summit.
*Values represented are the callus quality score means ± s.e. (n = 50). For comparison of callus quality score means for each callus treatment, significant differences are indicated with different letters ($P<0.05$).

To overcome the sub-optimal callus initiation from Tendercrisp plant tissues, agar solidified basal medium B5 containing 100mg/L serine, 2.5µM 2,4-D and 3.0µM kinetin was tested for its ability to induce viable embryogenic callus from petiole explants. Comparisons were made to two control media, B5 medium containing 100 mg/L serine without the inclusion of PGR, and MS basal medium containing 2.5 µM 2,4-D and 3.0 µM kinetin (Table 3.6). Due to bacterial and fungal infection of Summit petioles incubated on MS control medium, results were not generated for this control factor and are excluded from Table 3.6.

Tendercrisp and Summit petiole explants treated on B5 basal medium containing 100mg/L serine, 2.5 µM 2,4-D and 3.0 µM kinetin had a significantly greater callus quality score (3.12, $P < 0.05$) and percentage callus production (96%) than petioles of both cultivars incubated on all other treatment medium combinations tried in this study (Table 3.6). This treatment medium, for the induction of viable embryogenic callus from Tendercrisp petiole explants, was used for subsequent *A. tumefaciens* transformation experiments.

Table 3.6. Effect of basal media on callus production from petiole explants of celery cultivars Summit and Tendercrisp.

	Callus quality score *		Callus production (%) *+	
	Summit	Tendercrisp	Summit	Tendercrisp
Treatment^				
B5 control	0.22 ± 0.07a	0.52 ± 0.08a	0 ± 0a	0 ± 0a
B5 treatment	2.0 ± 0.19b	3.12 ± 0.17c	70 ± 31b	96 ± 8.9c
MS control	infected♣	1.94 ± 0.13b	infected	20 ± 9.1b

*Values represented are treatment means + s.e. (n = 39 for Summit B5 control; n= 50 for all other treatments). ^B5 control: B5 salts and vitamins, 100mg/L serine; B5 treatment: B5 medium, 100mg/L serine, 2.5μM 2,4-D, 3.0μM kinetin; MS control: MS medium, 100mg/L serine, 2.5μM 2,4-D, 3.0μM kinetin. +Callus production as a percentage of total petiole explants treated. For comparison of treatment means for each callus response, significant differences are indicated with different letters ($P<0.05$). ♣ No results generated due to microbial infection of summit petioles.

The Summit petioles produced callus with a mean quality score of 2.0 when grown on B5 callus initiation medium containing 100mg/L serine, 2.5 μM 2,4-D and 3.0 μM kinetin, and was higher than mean callus generated from petioles grown on control media (Table 3.6). However, this result was lower than the result obtained when Summit petioles were grown on MS solidified medium containing 0.25 μM 2,4-D and 5.0 μM kinetin (Figure 3.6).

3.3.4 Callus regeneration

To determine the optimal callus regeneration medium for the formation of viable plantlets from somatic embryos, callus explants for this experiment were derived from optimal callus initiation treatments for Summit and Tendercrisp, as defined in previous sections. At day 7, following the movement of callus to a range of callus regeneration media, callus was beginning to show changes in morphology (Table 3.7). Callus on treatment medium with no PGR and treatment medium containing 1.0 μM kinetin, of both celery cultivars derived from petiole, basal cotyledon and hypocotyl explants were beginning to form highly embryogenic callus forms, with individual embryos at varying developmental stages visible under magnification (Figure 3.7 I). Callus on control medium containing 0.25 μM 2,4-D showed similar cellular proliferation to callus explants on treatment medium but displayed no signs of embryo formation. Callus also began producing root protrusions when moved to control regeneration medium and a 16h/8h light/dark photoperiod (Figure 3.7 II)

The removal of PGR from regeneration medium or supplementation of regeneration medium with 1 μM kinetin increased the conversion of embryos to 'seedlings' at day 35 following movement of callus explants to regeneration medium (Figure 3.7 III). Summit and Tendercrisp callus generated from petiole explants cultured on regeneration medium excluding PGR showed the highest capacity for somatic embryogenesis and the production of well formed plantlets (Table 3.7). Generally, plantlets generated from callus cultured on medium containing 1 μM kinetin were vitrified and malformed. Limited success was achieved with seedling-derived cotyledon and hypocotyl explants on PGR-free medium. Overall, callus derived from

seedling roots performed poorly, neither proliferating nor producing embryogenic forms. Plantlets generated from callus could be transferred into the soil to grow into a mature plant.

Table 3.7. Effect of plant growth regulator treatment on the regeneration from Summit (S) and Tendercrisp (T) callus explants (derived from various tissue sources) of viable plantlets.

			Explant				
			Petiole	Apical Cotyledon	Basal Cotyledon	Hypocotyl	Root
Treatment							
0.25 µM 2,4-D (control)	7 d	S	+++	+++	++	+++	+
		T	+++	++	++	+++	++
	35 d	S	No	No	No	No	No
		T	No	No	No	No	No
no PGR	7 d	S	+++++*	+++	+++*	+++	++
		T	+++++*	++++*	++++*	+++	++
	35 d	S	Yes	Yes	Yes	No	No
		T	Yes	Yes	Yes	No	No
1 µM kinetin	7 d	S	++++*	+++	+++	++	+
		T	+++++*	++++	+++	+++*	+
	35 d	S	Yes	Yes	No	No	No
		T	Yes	No	No	Yes	No

Callus proliferation: +, callus explants remaining intact, no expansion; +++++, callus explants highly friable/crumbly, much proliferation. * Indicates presence of torpedo or heart shaped embryogenic forms. Somatic embryogenesis and formation of plantlets: Yes/No

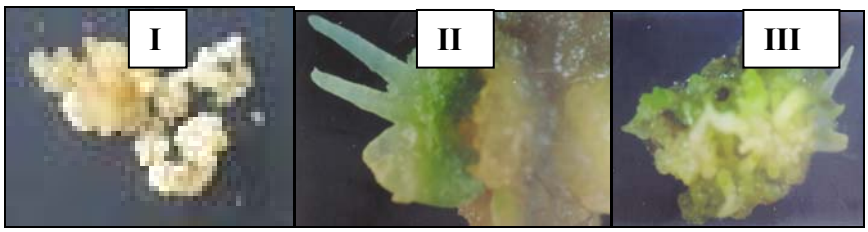

Figure 3.7. Effect of regeneration medium on callus cultures of celery cultivars Summit and Tendercrisp. I. Proliferating, friable Summit callus 7 days following movement to PGR-free regeneration medium. II. Tendercrisp callus displaying root protrusions following growth on control regeneration medium (0.5 µM 2,4-D). III. Conversion of Summit somatic embryos to plantlets 35 days following movement to PGR-free medium.

3.4 Discussion

As an initial step towards the development of sustainable tissue culture processes for celery cultivars Summit and Tendercrisp, the effect of different cytokinin treatments on meristematic proliferation from celery seedling shoot tips was assessed. This was to provide a means of clonal multiplication in a relatively short time for the maintenance of transgenic or non-transgenic clonal lines. It was initially thought the cytokinin treatment (5 µM BAP) most commonly applied for clonal proliferation of strawberries would be successful for the proliferation of celery plants. Results indicate this not to be the case, with seedling shoot tips incubated on this medium producing stunted plants, with no indication of newly generated plants from the meristematic base. The only treatment to produce a plant of normal form was that containing 5 µM kinetin. However this treatment did not give rise to meristematic proliferation of celery seedlings. Further investigation into other cytokinin analogues may reveal an optimal treatment for the proliferation of new plants from basal meristematic tissue of celery cultivars.

One of the most important hurdles towards the development of an efficient somatic embryogenic system is the induction of viable embryogenic callus from plant tissue. The PGR requirement of Summit and Tendercrisp for production of viable embryogenic callus differed greatly between cultivars. The requirement of Tendercrisp petioles for 2,4-D and kinetin in similar ratios (2.5 µM 2,4-D and 3.0 µM kinetin) for the production of embryogenic callus closely resembles results reported for other celery cultivars (Williams and Collin, 1976; Zee and Wu, 1979; Nadel *et al*. 1989, 1990a; Saranga and Jannick, 1991; Onishi *et al*., 1992 and Evanor *et al.*, 1994).

The PGR requirement of Summit seedling and petiole derived explants for the production of embryogenic callus differed from Tendercrisp. For optimal callus production, Summit explants performed best on medium containing 0.25 µM 2,4-D in combination with 5.0 µM kinetin. Favourable results were also obtained for seedling-derived explants on treatment medium containing 0.25 µM 2,4-D with 0.5 µM BAP or 5.0 µM BAP and petiole-derived explants on treatment medium containing 0.25 µM 2,4-D with 5.0 kinetin, 0.5 µM BAP or 5.0 µM BAP.

Tendercrisp petiole-derived explants performed better on B5 basal medium (Gamborg *et al.* 1968) when compared to MS basal medium (Murashige and Skoog 1962). B5 contains a much lower concentration of inorganic ammonium (1.01 mM $(NH_4)2SO_4$) compared to MS medium (20.06 mM NH_4NO_3) and it is thought in some instances that concentrations of ammonium above 10 mM may retard embryogenesis (Masuda *et al*. 1981). B5 medium has been used in both reports of celery transformation (Catlin *et al*. 1988; Liu *et al.* 1992). The inclusion of protein hydrolysates such as serine and casein hydrolysate in nutrient medium was also reported in both incidences. Catlin *et al*. (1988) augmented callus initiation medium with 100 mg/L serine, while Liu *et al*. (1992) included 100mg/L casein hydrolysate in callus initiation medium.

The use of petiole explants derived from stock plant cultures of cultivars Summit and Tendercrisp maintained *in vitro* avoids the requirement for fresh seedling material, providing a continual supply of sterile plant material for tissue culture and transformation experiments. A comparison of the callus derived from seedling and

petiole explants on their respective optimal treatment media indicates callus generated from petioles to possess a higher mean callus score and therefore it was concluded that petiole tissue had greater embryogenic potential.

Following the induction of viable embryogenic callus on petiole tissue, callus explants were moved to callus maintenance medium for proliferation. High 2,4-D levels are inhibitory to embryogenesis. However, lower levels of 2,4-D used in callus maintenance medium can stimulate embryogenesis and may be a factor in helping maintain the stability of the callus (Williams and Collin 1976). The removal of all PGRs or a reduction of the auxin component of plant nutrient medium will stimulate the germination of somatic embryos (Fujimura and Komamine 1979; Michalczuk *et al.* 1992). Reports of celery regeneration via somatic embryogenesis support this view. The removal of PGRs from basal medium has enabled the regeneration of plantlets from callus derived from petiole explants of Summit and Tendercrisp celery cultivars. Regeneration has similarly been achieved for petiole (Williams and Collin 1976b) and leaf (Saranga and Janick 1991) explants. Removal of 2,4-D while maintaining reduced kinetin concentration also enabled plantlet regeneration from callus explants of celery cultivars tested. This corresponds to previous reports where removal of auxins and reduction of cytokinin presence resulted in the successful transition from callus through somatic embryogenesis to plantlets (Williams and Collin 1976a; Nadel *et al*. 1989; Nadel *et al*. 1990a, 1990b).

In the celery somatic embryogenesis system presented in this chapter, callus was obtained 4 weeks after the explant was first placed on nutrient medium. Embryos were already forming at this stage and callus continued to initiate embryos during subculture. Following an 8-week callus maintenance and proliferation period, with 4-weekly subculture, callus was moved to a PGR-free regeneration medium in light conditions and embryos had germinated and produced seedlings with defined cotyledons and roots within 5 weeks. Although callus explants were maintained for 8-weeks to ensure an adequate number of regenerating embryos, reducing the maintenance and embryo proliferation period would reduce the time required to produce new plants from the original parent plant if smaller plant numbers was acceptable. Alternatively, increasing the maintenance and proliferation period would also provide larger plant numbers but increase the period before plants could be obtained following proliferation.

The results of this work provide detailed information on high throughput somatic embryogenesis in celery cultivars Summit and Tendercrisp, which will enable the production of transgenic celery lines. Celery petiole explants will be inoculated with *Agrobacterium tumefaciens* carrying reporter genes and antiviral ihpRNA cassettes and transformed cells subsequently regenerated via somatic embryos using the optimal methods and media identified in this chapter.

3.5 References

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4.0 *Agrobacterium*-mediated transformation of carrot and celery cultivars using reporter genes GFP and GUS

4.1 Introduction

The introduction of foreign DNA into plant cells and its subsequent expression has been widely applied for the genetic manipulation of plant phenotypes for the development of cultivars with traits of commercial interest (Melchers and Stuiver 2000). A variety of techniques exist for the direct introduction of new genetic material into plant host cells, including electroporation (Fromm *et al.* 1984), chemicals that increase uptake of DNA (Paszkowski *et al.* 1984), microinjection (Crossway *et al*. 1985) and DNA delivery via microparticle bombardment (Klein *et al.* 1987). However, it is widely believed that *A. tumefaciens*-mediated transformation has distinct advantages over direct transformation methods (Horsch *et al*. 1985; Riva *et al*.1998). This method has a higher transformation efficiency (Pawlowski and Somers 1996; Gelvin 1998) and transgenic plants possess a low transgene copy number and therefore fewer rearrangements (Flavell 1994; Koncz *et al.* 1994; Hansen *et al*. 1997; Vaucheret *et al*. 1998). Optimising the *A. tumefaciens*-plant cell interaction is considered to be the most important aspect for the development of efficient transformation protocols and the recovery of whole transgenic plants (Riva *et al*. 1998).

Carrot has been reported to be susceptible to infection by *Agrobacterium* wild type strains and disarmed strains of *A. tumefaciens* (De Cleene and De Ley 1976). From the late 1980s, carrot tissues have been routinely transformed with *A. tumefaciens* strains (Ryder *et al*. 1985; Scott and Draper 1987; Thomas *et al*. 1989; Wurtele and Bulka 1989; Liu *et al.* 1992; Pawlicki *et al*. 1992; Gilbert *et al*. 1996; Hardegger and Sturm 1998; Tang and Sturm 1999). The propensity of carrots for somatic embryogenesis (Quiros 2001) and its wide application in transformation systems has more recently seen the development of transgenic carrot genotypes with resistance against *Erysiphe heraclei* and *Alternaria dauci* (Takaichi and Oeda 2000) herbicide tolerance (Chen and Punja 2002), resistance to microbial, fungal and viral infection (Zheng *et al*. 2002) and tolerance to *Botrytis cinerea* and *Sclerotinia sclerotiorum* infection (Chen and Punja 2002; Punja and Chen 2004). Although reports of carrot transformation are widely published and provide a sound basis for transformation protocols, the development of cultivar specific protocols require the determination of optimal conditions for co-cultivation and regeneration and a useable selectable marker.

Unlike carrot, reports of celery transformation in scientific literature are limited (Catlin *et al*. 1988; Liu *et al*. 1992). Although both sources claim celery is amenable to *Agrobacterium*-mediated transformation, more extensive investigations into transformation efficiency of target cultivars using a range of reporter genes and *A. tumefaciens* strains have yet to be carried out.

To improve the T-DNA-mediated transformation frequency of economically important carrot and celery crops, the effects of *A. tumefaciens* strains LB4404 and AGL1 carrying the reporter genes green fluorescing protein (GFP) and β-glucuronidase (GUS) have been investigated. Due to the limited reports of celery transformation, the optimisation of celery transformation protocols has also involved

testing different growth media and plant growth regulator (PGR) combinations and concentrations (as defined in Sections 3.3.3.2 and 3.3.3.3) for their ability to induce the formation and regeneration of transgenic callus. Carrot plants were regenerated via somatic embryogenesis on agar solidified medium based on optimal regeneration protocols defined in Sections 3.3.3.2 and 3.3.3.3. GFP and GUS expression was visually assessed in callus and plant tissue using microscopy and histochemical assays respectively. The influence of the antibiotics kanamycin and hygromycin on carrot and celery callus formation was also examined to determine the optimal antibiotic and antibiotic concentration for control of non-transgenic escapes while maintaining plant tissue capacity for callus initiation. The kanamycin resistance protein is encoded by the *nptII* gene and this gene is located within the intron-containing hairpin (ihp) RNA construct that will be used for the generation of virus resistant transgenic carrot and celery genotypes. Transgenic plantlets that are resistant to kanamycin will contain the ihpRNA construct that confers virus resistance. The effects of hygromycin on carrot and celery callus initiation was useful for comparison with the effects of kanamycin. If kanamycin proves highly toxic to carrot and celery plant tissue, ihpRNA vectors may require altering to include a selectable marker such as hygromycin and therefore the effects of this antibiotic on carrot and celery initiation was deemed necessary.

4.2 Materials and methods

4.2.1 Plant materials

Four-week-old petiole material of carrot (*Daucus carota* var. *sativum*) cultivars Crusader and Stefano and of celery (*Apium graveolens* var. *dulce*) cultivars Summit and Tendercrisp was used as a source of explant material for transformation experiments. Petiole material, derived from stock plant cultures maintained on agar-solidified, plant growth regulator (PGR) free MS medium (Murashige & Skoog 1962) at 25ºC with a 16h/8h light/dark photoperiod, was cut into 1 cm sections immediately prior to immersion in *A. tumefaciens* cultures.

4.2.2 Standardisation of transformant selection

The influence of the antibiotics kanamycin and hygromycin on callus formation was tested. For antibiotic sensitivity tests, carrot and celery petiole explants were cultured on callus induction medium (CI) (Carrot: MS + 0.5 µM 2,4-D; Celery: MS + 0.25 µM 2,4-D + 2.5 µM kinetin) containing 25, 50, 100 and 200 µg/mL kanamycin and 5, 10, 25 and 50 µg/mL hygromycin. As a control petioles were also cultured on callus induction medium without antibiotics. The antibiotic sensitivity trial was conducted with 5 repetitions of 10 explants each for each antibiotic variable. Following a four week period on callus induction medium, callus was assessed based on the callus grading system as described in Section 2.2.3.2 and 3.2.3.1 for carrot and celery respectively.

4.2.3 Bacterial strains and vectors

Two reporter genes and two *A. tumefaciens* strains were used in this study to visually determine transformation efficiency in carrot and celery cells: *A. tumefaciens* strains, LBA4404 and AGL1 harbouring the GFP gene and GUS gene (Table 4.1).

Table 4.1. The combination of binary plasmids and *Agrobacterium tumefaciens* strains used for transformation of carrot and celery tissue together with the corresponding antibiotics used for bacterial and plant selection

	Binary plasmid			
	GFP5intron	**GFP5Δ(cyto)**	**400GUSintron**	**35SGUSintron**
A. tumefaciens strain	LBA4404	AGL1	LBA4404/ AGL1	LBA4404
Bacteria selection[1]	50 Rif and 25 Chlor	100 Amp and 50 Rif	50 Rif and 50 Spec	50 Rif and 50 Kan
Plant selection[1]	50 Kan	100 Kan	100 Kan	100 Kan

[1]All concentrations in µg/mL. Abbreviations: Rif, rifampin; Chlor, chloramphenicol; Kan, kanamycin; Amp, ampicillin; Spec, spectinomycin.

The binary plasmids used in this study contain a GUS gene that has a portable intron in its codon region that prevents GUS activity in *A. tumefaciens* cells (Vancanneyt *et al*. 1990). Likewise, the GFP5intron binary plasmid contains an intron in the codon region, to prevent GFP expression in bacteria. The T-DNA of all binary vectors contains the *nptII* gene, conferring kanamycin resistance to transformed plant cells.

4.2.4 Growth and induction of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strains were maintained on YEP selection plates(10 g/l Peptone, 10 g/L Yeast extract, 5 g/L NaCl and 10-15 g/L Agar, pH 7.5) at 4ºC under selection as listed in Table 4.1. All *A. tumefaciens* cultures used for transformation were induced by acetosyringone before co-cultivation with plant tissues. Two to three loops of fresh bacterial colony was grown in 20 ml YEP medium containing the appropriate bacterial antibiotic selection (Table 4.1) for 24 h at 28ºC in the dark with rotary agitation (200 rpm). *A. tumefaciens* cultures were centrifuged for 5 min at 5000 rpm, washed with sterile 0.9% NaCl, centrifuged again, then resuspended in 100 ml induction medium (0.5% glucose; 1 x AB salts (20 g/L NH_4Cl, 6 g/L $MgSO_4.7H_20$, 3g/L KCl, 264 mg $CaCl_2.2H_20$ and 50 mg $FeSO_4.7H_20$); 30 mM MES; 2 mM sodium phosphate; 100 µM acetosyringone; pH 5.6). Following incubation for 16-18 h at 28ºC in the dark with rotary agitation (100 rpm), *A. tumefaciens* cultures were applied to petiole explant material.

4.2.5 Plant transformation and regeneration

4.2.5.1 *Daucus carota* var. *sativum* (Carrot)

Petioles from carrot (*Daucus carota* var. *sativum)* stock plants, 4 weeks following subculture, were cut into sections 1 cm in length and immersed in a solution of induced *A. tumefaciens* for 10 min in a Petri dish. Following infection, petiole sections were blotted dry on sterile paper napkins and placed on carrot co-cultivation (*Dc*CC) medium (Table 4.2). The explants were co-cultured in the dark for three days at 25ºC. After co-cultivation the explants were moved to carrot callus initiation medium with pre-selection (*Dc*CI-PS) (Table 4.2) for 4 weeks. Following the callus induction period callus was removed from those explants with proliferating callus at either one or both ends of petiole sections and transferred to carrot callus maintenance medium with selection (*Dc*CM-S) (Table 4.2) for 8 weeks. Callus transferred to CM

medium was put under kanamycin selection of 25 µg/mL for 4 weeks then selection was increased to 50 µg/mL for a further 4 weeks.

Following the 8 week callus maintenance and proliferation period kanamycin resistant callus was moved to PGR free basal medium with selection (*Dc*R-S) and cultures moved to a 16/8 light/dark photoperiod for regeneration from somatic embryos (Table 4.2). Shoots with defined cotyledons and roots that were emerging from callus cultures as a result of somatic embryogenesis, were placed on agar solidified MS medium containing 150 µg/mL Timentin ™ and 50 µg/mL kanamycin and maintained at 25ºC with a 16h/8h light/dark photoperiod for further development. Transgenic plants were routinely subcultured every 8 weeks.

Table 4.2. The composition of the tissue culture media used for *A. tumefaciens*-mediated transformation and regeneration from carrot petiole explants.

	Media formula			
	*Dc*CC	*Dc*CI-PS	*Dc*CM-S	*Dc*R-S
MS salts and vitamins (g/L)	4.4	4.4	4.4	4.4
2,4-D (µM)	-	0.5	0.5	-
Sucrose (g/L)	30	30	30	30
Agar (g/L)	8	8	8	8
pH	5.6	5.6	5.6	5.6
Kanamycin (µg/mL)	-	-	25/50	50
Timentin (µg/mL)	-	150	150	150

Abbreviations: *Dc*, *Daucus carota*; CC, co-cultivation medium; CI, callus induction; PS, pre-selection medium; S, selection medium; CM, callus maintenance medium; R, regeneration medium; MS, Murashige and Skoog (1962); 2,4-D, 2,4-dichlorophenoxyacetic acid.

4.2.5.2 *Apium graveolens* var. *dulce*

For the regeneration of celery plants via somatic embryos following transformation with *A. tumefaciens* strains, two alternative regeneration systems were explored, (I) and (II). Petioles from celery stock plants, 4 weeks following subculture, were cut into sections 1 cm in length and immersed in a solution of induced *A. tumefaciens* for 10 minutes in a Petri dish. Following infection, petiole sections were blotted dry on sterile paper napkins and placed on celery co-cultivation (*Ag*CC-I or *Ag*CC-II) media (Table 4.3). The explants were co-cultured in the dark for three days at 25ºC. After co-cultivation the explants were moved to celery callus induction medium (*Ag*CI-PS) (I) or (II) (Table 4.3). At this stage, those explants on *Ag*CI-PS (I) remained without kanamycin selection for 1 week before being moved to *Ag*CI-S (I) containing 50 µg/mL kanamycin for a further 3 weeks. Those explants on *Ag*CI-PS (II) medium were transferred to light with a 16h/8h light/dark photoperiod for the duration of transformation experiments and remained without kanamycin selection until the callus maintenance phase. Following the callus induction period, callus protrusions were removed from those explants with proliferating callus at either one or both ends of petiole sections. The callus protrusions were transferred to celery callus maintenance and proliferation media *Ag*CM-S (I) or (II) for 8 weeks (Table 4.3). Callus transferred

to *Ag*CM-S (I) was moved to fresh *Ag*CM-S (I) medium after 4 weeks. Callus transferred to *Ag*CM-S (II) was put under kanamycin selection of 25 µg/mL for 4 weeks then selection was increased to 50 µg/mL for a further 4 weeks.

Following the 8 week callus maintenance and proliferation period, kanamycin resistant celery callus was moved to PGR free basal medium with selection (AgR-S (I) or (II)) (Table 4.3). At this stage those cultures on AgR-S (I) medium, which until this stage had been proliferating in the dark, were moved to a 16/8 hour light/dark photoperiod for regeneration from somatic embryos (Table 4.2). Shoots with defined cotyledons and roots that were emerging from callus cultures as a result of somatic embryogenesis, were placed on agar solidified MS medium containing 150 µg/mL Timentin ™ and 50 µg/mL kanamycin and maintained at 25ºC with a 16h/8h light/dark photoperiod for further development. Transgenic plants were routinely subcultured every 8 weeks.

Table 4.3. The composition of tissue culture media used for celery transformation and regeneration from petiole explants.

	Media formula									
	*Ag*CC		*Ag*CI-PS		*Ag*CI-S		*Ag*CM-S		*Ag*R-S	
	(I)	(II)	(I)	(II)	(I)	(II)	(I)	(II)	(I)	(II)
MS salts and vitamins (g/L)	4.4	-	4.4	-	4.4	-	4.4	-	4.4	-
B5 salts and vitamins (g/L)	-	3.2	-	3.2	-	3.2	-	3.2	-	3.2
2,4-D (µM)	-	-	0.25	2.5	0.25	2.5	0.5	0.5	-	-
Kinetin (µM)	-	-	2.5	3.0	2.5	3.0	0.5	0.5	-	-
Serine	-	100	-	100	-	100	-	100	-	100
Sucrose (g/L)	30	30	30	30	30	30	30	30	30	30
Agar (g/L)	8	8	8	8	8	8	8	8	8	8
pH	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6
Kanamycin (µg/mL)	-	-	-	-	50	-	50	25/ 50	50	50
Timentin (µg/mL)	-	-	150	150	150	-	150	150	150	150

Abbreviations: *Ag*, *Apium graveolens*; CC, co-cultivation medium; CI, callus induction; PS, pre-selection medium; S, selection medium; CM, callus maintenance medium; R, regeneration medium; MS, Murashige and Skoog (1962); B5, Gamborg *et al*. (1968); 2,4-D, 2,4-dichlorophenoxyacetic acid.

4.2.6 Determining transformation efficiency

4.2.6.1 β-glucuronidase histochemical assays

The β-glucuronidase activity was determined in callus and plant tissue by histochemical assays. β-glucuronidase activity was assessed at 2, 4, 6, 9, 12 and 15 weeks following inoculation of petioles with *A. tumefaciens* strains, depending on the amount of material available. At these intervals, the frequency of T-DNA transfer (%) was defined as the number of callus explants displaying blue spots (indicating β-glucuronidase activity), relative to the total number of explants assayed at any one time. Total transformation %, as calculated at 6 and 15 weeks and was defined as the

number of callus or plant tissue with β-glucuronidase activity relative to the total number of petiole explants inoculated and which survived the selection process.

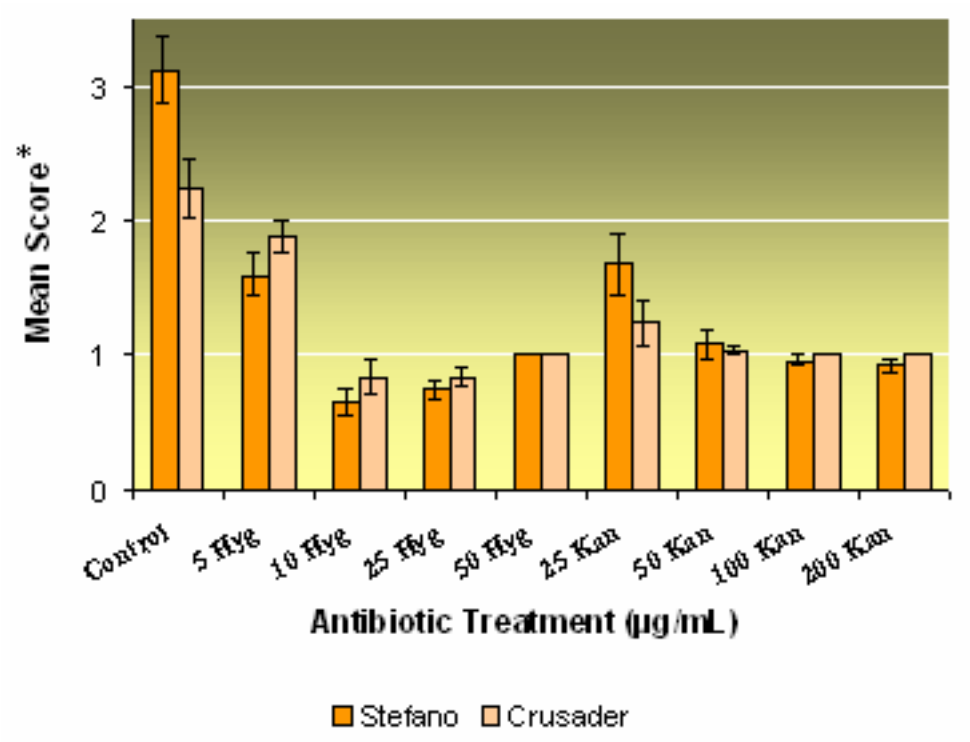
4.2.6.2 GFP visualisation

GFP activity was determined in callus tissue by visualisation on a Leica compound microscope with a UV light source and GFP filter. GFP activity was monitored at 2, 4, 6, 9, 12 and 15 weeks following inoculation of petioles with *A. tumefaciens* strains depending on the amount of material available. At these intervals, the frequency of T-DNA transfer (%) was defined as the number of callus explants displaying fluorescing spots, relative to the total number of explants assayed at any one time. Total transformation percentage was assessed at 6 and 15 weeks and was defined as the number of callus or plant tissue with GFP activity relative to the total number of petiole explants inoculated (minus infected explants).

4.3 Results

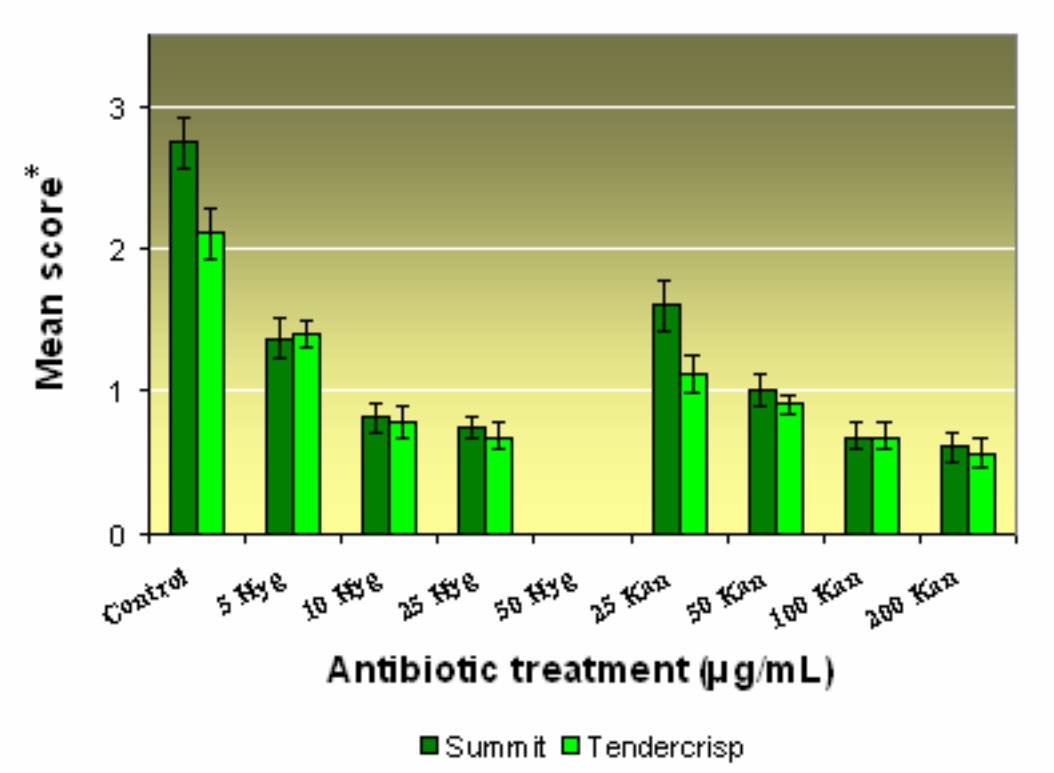
4.3.1 Standardisation of transformant selection

The sensitivity of carrot and celery tissue to kanamycin and hygromycin (the antibiotics used as selectable markers in plant transformation procedures) were assayed by culturing petiole segments on DcCI and Ag-CI media containing kanamycin at concentrations ranging from 0 – 200 µg/mL and hygromycin at concentrations ranging from 0 – 50 µg/mL (Figures 4.1; Figure 4.2).



*Values represented are callus quality score means ± s.e.

Figure 4.1. Effect of varying concentrations of the antibiotics Hygromycin (Hyg) and Kanomycin (Kan) on callus production (defined by callus quality score) from petiole explants of carrot cultivars Stefano and Crusader.



*Values represented are callus quality score means ± s.e.

Figure 4.2. Effect of varying concentrations of the antibiotics Hygromycin (Hyg) and Kanomycin (Kan) on callus production (defined by callus quality score) from petiole explants of celery cultivars Summit and Tendercrisp.

The concentration of kanamycin and hygromycin in callus initiation medium affected the yield and quality of callus from both carrot and celery petioles (Figures 4.1 and 4.2). Callus formation from petiole explants of carrot cultivars Stefano and Crusader and celery cultivars Summit and Tendercrisp stopped at hygromycin concentrations of 10 µg/mL, which is five- to tenfold lower than the growth-inhibitory concentrations of kanamycin (50 - 100 µg/mL). Carrot and celery petiole explants grown in the absence of kanamycin or hygromycin (control treatment) produced callus with a higher mean callus quality score. Only kanamycin at 25 µg/mL and hygromycin at 5 µg/mL allowed for some degree of callus production from carrot and celery petioles.

4.3.2 Efficiency of *Agrobacterium*-mediated T-DNA transfer to carrot and celery cells

4.3.2.1. Daucus carota var. sativum

The efficiency of T-DNA transfer to carrot petiole tissue was determined with two strains of *A. tumefaciens* harbouring GUS and GFP reporter genes. Petiole explants inoculated with GUS-containing *A. tumefaciens* strains were transformed at a very low rate (Table 4.4). The highest transformation efficiency was obtained from Crusader petioles inoculated with LBA4404 strain of *A. tumefaciens* containing the 35SGUSintron. Callus initiated from Crusader petiole explants began showing signs of GUS activity at 2 weeks post inoculation (wpi) (Figure 4.3.I). At 6 wpi, 11% of callus explants assayed were showing blue spots. All callus explants of the Crusader petioles inoculated with the LBA4404 strain of *A. tumefaciens* containing the

35SGUSintron were assayed at 6 wpi, and therefore there were no results recorded at 15 wpi for this strain/construct combination. The LBA4404 strain of *A. tumefaciens* containing either the 400GUSintron or the 35SGUSintron were equally as efficient for the transformation of Crusader petioles, with 10% of callus explants showing signs of GUS activity at week 6 following inoculation. At week 15 following inoculation with the LBA4404 strain of *A. tumefaciens* containing the 400GUSintron, when all remaining explants were assayed, the number of callus explants expressing GUS had not increased. Therefore the transformation efficiency as a percentage of total explants inoculated has decreased to 2%. There was no observed increase in transformation events 6 weeks following inoculation of Stefano petioles with the AGL1 strain of *A. tumefaciens* containing the 400GUSintron and only one additional transformation event for Crusader petioles inoculated with the AGL1 strain of *A. tumefaciens* containing the 400GUSintron 6 weeks post inoculation was recorded. For petiole explants transformed with AGL1 strain of *A. tumefaciens* containing the 400GUSintron, GUS expression was evident at 2 wpi for both Stefano and Crusader (Figure 4.3.I and II) and transformation efficiency was 5% at 6 wpi. At 15 wpi, transformation efficiency as a percentage of total explants inoculated, was 1% for Stefano and 1.3% for Crusader.

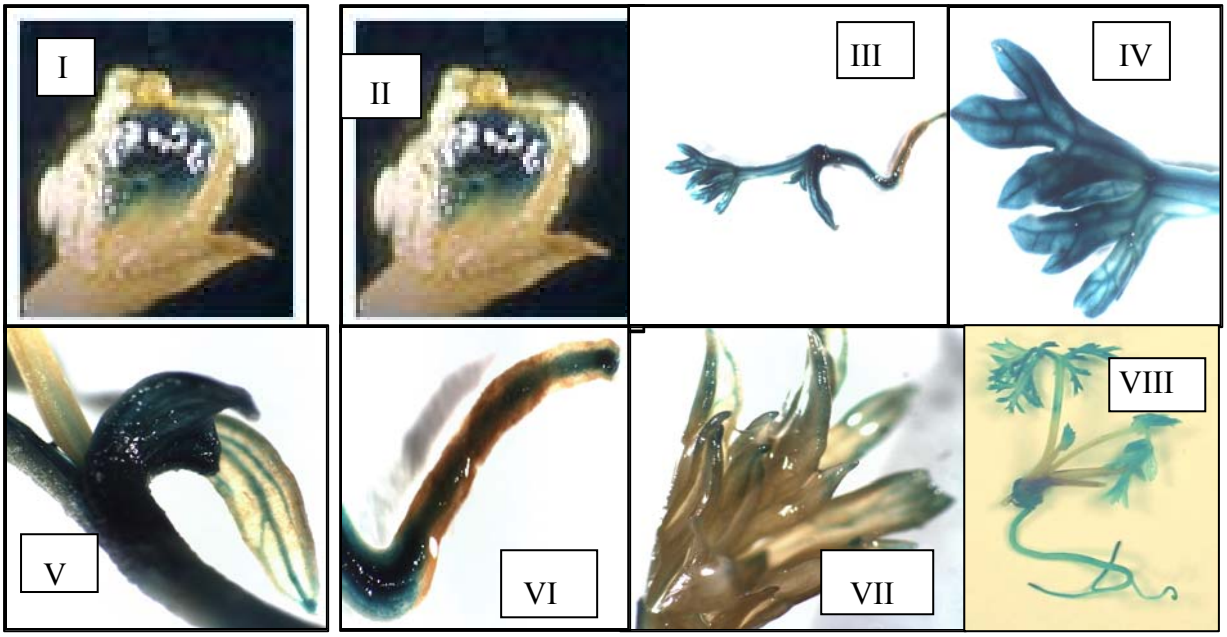


Figure 4.3. Carrot tissue displaying β-glucuronidase activity following histochemical assay. I. Stefano petiole explant producing callus. II. Crusader petiole explant producing callus. III. Newly germinated Crusader somatic embryo. IV. Close up of first true leaves. V. Close up of cotyledon/hypocotyl junction. VI. Close up of primary root. VII. Sub-cultured Crusader stock plant. VIII. Newly germinated Crusader embryo of unnatural form.

Table 4.4. Transformation of carrot petioles by *A. tumefaciens* strains LBA440 and AGL1 harbouring either GFP or GUS reporter genes.

Co-cultivation conditions				**Number of explants transformed**							
Binary plasmid	*Agro.* strain	Cultivar	No. explants inoculated	2 wpi	4 wpi *Dc*CM-S	6 wpi	Total 1	9 wpi	12 wpi *Dc*R-S	15 wpi	Total 2
GFP5intron	LBA4404	Stefano	111	107 (111)*	107 (111)	107 (111)	107/111 (96%)	36 (108)	57 (108)	-	57/108 (53%)$^{+}$
		Crusader	50	0 (50)	9 (50)	25 (50)	25/50 (50%)	25 (50)	-	-	25/50 (50%)
GFP5Δ(cyto)	AGL1	Stefano	300	0 (300)	97 (300)	100 (300)	100/300 (33%)	-	-	-	100/300 (33%)
		Crusader	300	0 (300)	123 (300)	142 (300)	142/300 (47%)	-	-	-	142/300 (47%)
35SGUSintron	LBA4404	Stefano	-	-	-	-	-	-	-	-	-
		Crusader	164	3 (32)	9 (69)	6 (63)	18/164 (11%)	-	-	-	-
400GUSintron	LBA4404	Stefano	-	-	-	-	-	-	-	-	-
		Crusader	300	3 (30)	-	3 (30)	6/60 (10%)	-	-	-	6/300 (2%)
400GUSintron	AGL1	Stefano	300	1 (30)	-	2 (30)	3/60 (5%)	-	-	-	3/300 (1%)
		Crusader	300	1 (30)	-	2 (30)	3/60 (5%)	-	-	1 (300)	4/300 (1%)

Abbreviations: wpi, weeks post inoculation; *Dc*CM-S, Callus removed from explants and placed on callus maintenance medium consisting of 0.5 µM 2,4-D and 25 µg/mL kanamycin; *Dc*R-S, Callus moved to regeneration medium consisting of MS medium containing 50 µg/mL kanamycin. * the number in parenthesis = the number of explants visualised/assayed. $^{+}$ The number in parenthesis = % number of transgenic calli or plants/no. petioles inoculated (minus infected explants).

Despite the movement of all callus cultures, resulting from transformation with the LBA4404 and AGL1 strains of *A. tumefaciens* containing the 400GUSintron combinations, to PGR-free medium and a 16h/8h light/dark photoperiod, only two GUS-positive Crusader plants successfully regenerated from transgenic callus (Figure 4.3.III-VI). Of the two Crusader plants regenerated from transgenic callus, one displayed abnormal form (Figure 4.3.VII) and subsequently died. The other GUS-positive plants displayed normal form and were readily sub-cultured to produce a transgenic population (Figure 4.3.VIII).

Inoculation of carrot petiole explants with *A. tumefaciens* strain/GFP transgene combinations resulted in a large number of GFP-expressing cells (Table 4.4). Callus initiated from Stefano petiole explants began showing signs of GFP activity at 2 wpi with the *A. tumefaciens* strain LBA4404 harbouring the intron-containing GFP5 gene construct (LBA4404/GFP5intron) (Figure 4.4.I). At 6 wpi, 96% of Stefano petiole explants inoculated with LBA4404/GFP5intron were showing spots of fluorescence when viewed under UV light (Figure 4.4.IV). This percentage decreased to 53% by week 15 indicating expression of GFP was either transient or toxic to plant cells.

Callus initiated from Crusader inoculated with LBA4404/GFP5intron began showing signs of GFP activity at week 4 following inoculation (4.4.II and III). At 6 wpi, 50% of the transformed petioles showing GFP activity when viewed under UV light (4.4.V) and this level of transformation efficiency was maintained until 15 wpi. For Stefano and Crusader inoculated with LBA4404/GFP5intron, all callus explants (both confirmed and unconfirmed transformants) were placed on callus regeneration medium (*Dc*R-S) at 12 wpi. Despite the removal of PGR from basal growth medium and the movement of explants to a 16h/8h light/dark photoperiod, callus failed to regenerate. Visualisation of GFP-positive callus under magnification indicated that callus was undergoing somatic embryogenesis due to the presence of embryogenic forms (Figure 4.4.VI), but the final developmental stages of embryogenesis were not witnessed.

Both carrot cultivars showed a decrease in transformation efficiency when inoculated with *A. tumefaciens* strain AGL1 harbouring the GFP5Δ construct (AGL1/GFP5Δ) (Table 4.4). AGL1/GFP5Δ was 3 times less effective than LBA4404/GFP5intron for the transformation of Stefano petioles at 6wpi. For the transformation of Crusader petioles, AGL1/GFP5Δ proved only slightly less effective than LBA4404/GFP5intron, with 47% of explants showing spots of fluorescence at 6wpi when viewed under UV light. Transformation efficiency remained constant until 15 wpi for both cultivars inoculated with AGL1/ GFP5Δ. The movement of all GFP-positive callus explants (both transformed and non-transformed) to PGR-free medium and a 16h/8h light/dark photoperiod, failed to initiate regeneration.

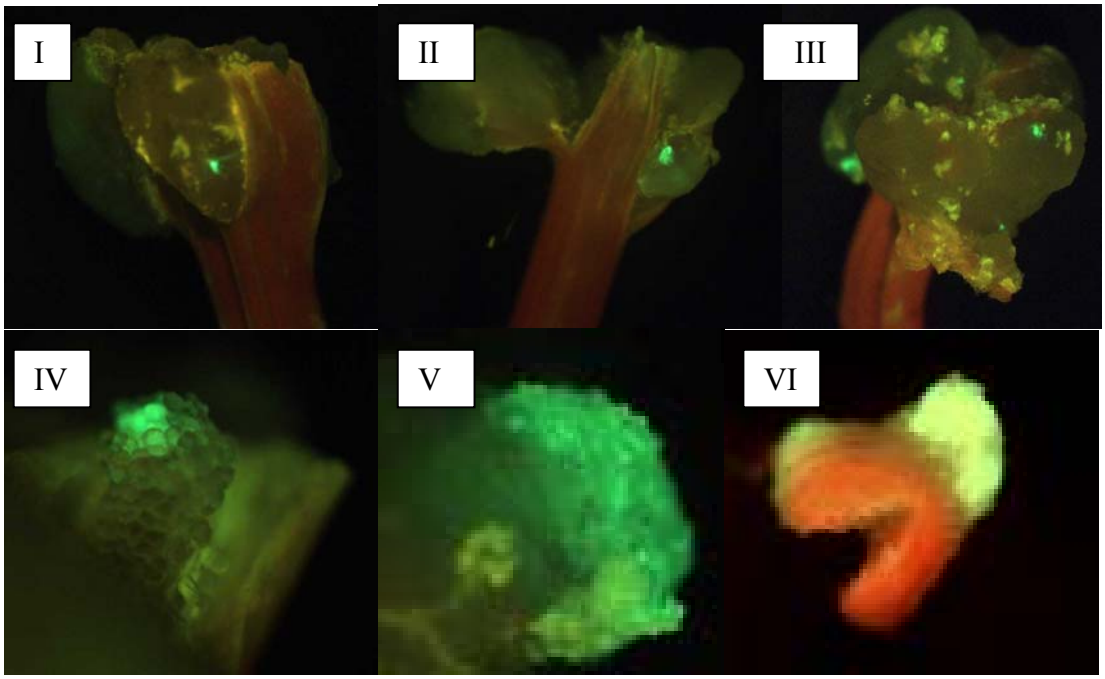


Figure 4.4. Carrot tissue displaying GFP activity following visualising under UV light. I. Stefano petiole explant producing callus. II. Crusader petiole explant producing callus. III. Crusader petiole explant producing callus with multiple fluorescing spots. IV. Close up of Stefano callus explant. V. Close up of Crusader callus explant. VI. Close up of Crusader heart-shaped embryo.

4.3.2.2. *Apium graveolens* var. *dulce*

The efficiency of the T-DNA transfer of the GUS and GFP reporter genes to celery petiole tissue via inoculation with two strains of *A. tumefaciens* (LBA4404 and AGL1) was determined. The transformation of celery petiole explants with *A. tumefaciens* strains containing the GUS transgenes was unsuccessful (Table 4.5). From a possible 2157 celery petiole explants transformed with the various *A. tumefaciens* strain/GUS gene constructs (ie. the LBA4404 strain of *A. tumefaciens* containing the 35SGUSintron and 400GUSintron constructs; and the AGL1 strain of *A. tumefaciens* containing the 400GUSintron construct), only one Tendercrisp petiole explant showed GUS expression when inoculated with LBA4404 harbouring the 35SGUSintron (Figure 4.5.I). Due to the destructive nature of the GUS histochemical assay, the progression of this GUS-positive callus through subsequent regeneration processes was not possible and therefore the efficiency of the plant regeneration systems I and II (Table 4.3) could not be assessed.

Inoculation of celery petiole sections with the LBA4404 and AGL1 strains of *A. tumefaciens* containing the GFP gene constructs resulted in a larger number of transformed cells when compared to the transformation efficiencies obtained when using the same *A. tumefaciens* strains containing the GUS gene constructs (Table 4.5). However, inoculation of Summit and Tendercrisp petioles with the AGL1 strain of *A. tumefaciens* harbouring the GFP5Δ construct resulted in no transformation events (Table 4.5). Summit petiole explants proved marginally more responsive than Tendercrisp petioles when transformation with the LBA4404 strain of *A. tumefaciens* containing the intron-containing GFP5 gene (LBA4404/GFP5intron). Summit

petioles began showing signs of GFP activity at week 4 following inoculation with LBA4404/GFP5intron and regeneration via system I (MS medium). At 6 wpi, 5% of Summit petiole explants inoculated with LBA4404/GFP5intron were showing spots of fluorescence when viewed under UV light. This percentage decreased to 1.4% by week 15, indicating expression of GFP was either transient or toxic to celery plant cells. Callus initiated from Summit petioles and inoculated with the same *A. tumefaciens* strain/GUS gene combinations but using regeneration system II (B5 medium) showed a higher percentage of GFP activity than callus generated using regeneration system I (Table 4.5). Summit petioles showed GFP activity at 2 wpi inoculation with LBA4404/GFP5intron and initial regeneration via system II (Figure 4.5.II). At 6 wpi 12% of Summit callus explants were showing spots of fluorescence (Figures 4.5.IV and V), before dropping back to 2% GFP activity at 15 wpi.

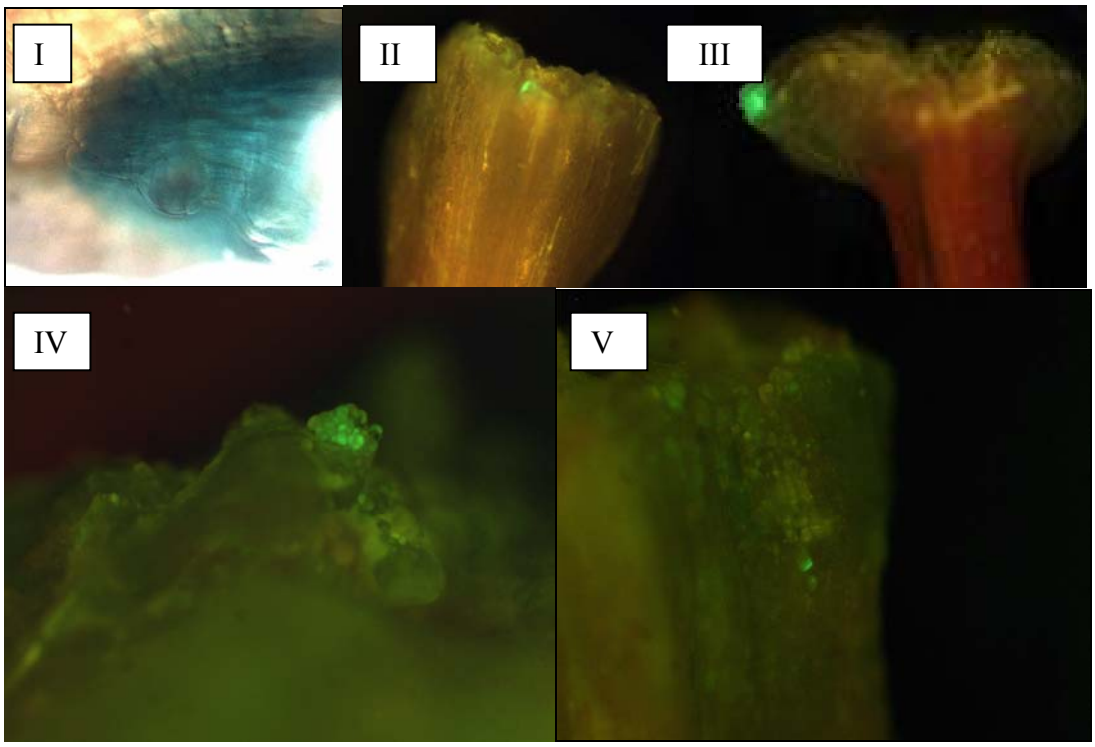


Figure 5.5. Celery tissue displaying β-glucuronidase and GFP activity. I. Tendercrisp petiole explant producing GUS +ve callus. II. Tendercrisp petiole explant with GFP +ve tissue. III. Summit petiole explant producing GFP +ve callus. IV. Close up of GFP +ve Summit callus explant. V. Close up of GFP +ve Summit callus explant with minimal fluorescence.

Table 5.5. Transformation of celery petioles by *A. tumefaciens* strains LBA440 and AGL1 harbouring GFP and GUS reporter genes.

Co-cultivation conditions					**No. explants transformed**							
Binary plasmid	*Agro.* Strain	Cultivar	No. explants inoculated	Regen. system	2 wpi	4 wpi *Ag*CM-S	6 wpi	Total 1	9 wpi	12 wpi *Ag*R-S	15 wpi	Total 2
GFP5intron	LBA4404	Summit	150	(I)	0 (150)	2 (147)	7 (147)	7/147 (5%)	-	-	-	-
			200	(II)	0 (200)	0 (200)	11 (93)	11/93 (12%)	15 (93)	2 (93)	-	2/93 (2%)
		Tendercrisp	150	(I)	0 (150)	0 (149)	4 (138)	4/138 (3%)				
			200	(II)	0 (200)	0 (200)	5 (67)	5/67 (7%)	6 (67)	0 (67)	-	0/67 (0%)
GFP5Δ(cyto)	AGL1	Summit	300	(II)	0 (300)	0 (300)	0 (300)	0/300 (0%)	-	-	-	-
		Tendercrisp	300	(II)	0 (300)	0 (300)	0 (300)	0/300 (0%)	-	-	-	-
35SGUSintron	LBA4404	Summit	627	(I)	0 (166)	0 (461)	-	0/627 (0%)	-	-	-	-
		Tendercrisp	33	(I)	0 (11)	1 (22)	-	1/33 (3%)	-	-	-	-
400GUSintron	LBA4404	Summit	300	(II)	0 (300)	0 (300)	0 (300)	0/300	-	-		
		Tendercrisp	300	(II)	0 (300)	0 (300)	0 (300)	0/300 (0%)	-	-		
400GUSintron	AGL1	Summit	300	(II)	0 (30)	3 (30)	0 (30)	3/300 (1%)	-	-		
		Tendercrisp	300	(II)	0 (30)	0 (30)	0 (30)	0/300 (0%)	-	-		

Abbreviations: wpi, weeks post inoculation; *Ag*CM-S, Callus removed from explants and placed on callus maintenance medium with selection as defined in Section 5.2.5.2; *Ag*R-S, Callus moved to regeneration medium with selection as defined in Section 5.2.5.2. * No. in parenthesis = no. explants visualised/assayed. $^{+}$ No. in parenthesis = no. transgenic calli or plants/no. petioles inoculated (minus infected explants) (as a percentage).

Callus initiated from Tendercrisp inoculated LBA4404/GFP5intron showed similar trends to those witnessed for Summit but with a lower transformation efficiency (Table 4.5). Callus initiated via regeneration system II (B5 medium) showed signs of GFP activity at 4 wpi (Figure 4.5.III), while callus initiated via regeneration system I (MS medium) did not display GFP activity until week 6. At 6 wpi, regeneration system II had produced more than twice the number of GFP-positive callus explants than regeneration system I. This percentage transformation efficiency decreased to 3% and 0% by week 15 for both regeneration systems I and II (Table 4.5). For Summit and Tendercrisp petioles inoculated with LBA4404/GFP5intron, all callus explants (confirmed transformed and unconfirmed transformed) were placed on callus regeneration medium (*Ag*R-S) 12 wpi. Despite the removal of PGR from basal growth medium and the movement of explants to a 16h/8h light/dark photoperiod, callus failed to regenerate. At 15 wpi callus was still in clump form and the proliferation and separation of embryogenic forms indicative of somatic embryogenesis was not evident.

To determine whether *A. tumefaciens* and/or components of the *A. tumefaciens* inoculation media (ie ecetosyrinogone) is toxic to the celery callus tissue a comparison of petiole explants inoculated with the two *A. tumefaciens* strains was made with celery petiole explants inoculated with water. At 2 wpi (prior to the addition of antibiotic selection to culture medium), there was visible evidence that the celery petiole explants were adversely effected by the presence of *A. tumefaciens* (Figure 4.6.I). Necrosis, beginning at the ends of petiole explants, and in some instances to cover entire explants, was seen on the petiole explants inoculated with *A. tumefaciens* and there was minimal evidence of callus proliferation at 2 wpi. The water inoculated petiole explants were beginning to produce callus at one or both ends. Some petiole explants that had been inoculated with *A. tumefaciens* produced sufficient callus for excision at 4 wpi. The resultant callus explants began producing root-like protrusions (Figure 4.6.II), but once root-like structures became evident, callus explants quickly degraded, becoming brown and eventually dying.

Figure 5.6. I. Comparison of Summit petioles 2 wpi: (top) water inoculation; (bottom) *A. tumefaciens* inoculation. II. Excised Summit callus explant 6 wpi, showing necrosis and root protrusions.

4.4 Discussion

A. tumefaciens-mediated DNA transfer, selection of transformed cells with the antibiotic kanamycin and plant regeneration via somatic embryogenesis are the preferred methods for the production of transgenic plants (Scott and Draper 1987; Thomas *et al*. 1989; Wurtele and Bulka 1989; Balestrazzi *et al.* 1991; Pawlicki *et al*. 1992; Hardegger and Sturm 1998) and the incorporation of commercially desirable traits (Gilbert *et al*. 1996; Punja and Raharjo 1996; Takaichi and Oeda 2000; Zheng *et al*. 2002; Marquet-Blouin *et al.* 2003; Park *et al*. 2004).

Chen and Punja (2002) compared the use of hygromycin with published reports of transformation frequency using kanamycin as a selectable marker (Gilbert et al. 1996; Pawlicki et al. 1992) and determined the transformation frequency was approximately one-half using hygromycin. The findings of this chapter showed that hygromycin severely affected the production of callus for both carrot and celery when compared to kanamycin (Figures 4.1 and 4.2).

Only one plant from the original 300 400GUSintron/AGL1 transformants was regenerated into a normal carrot plant (Figure 4.3 VII). The high transformation efficiency seen using the LBA4404/35SGUSintron combination (table 4.4), compared with transformation efficiencies from other *A. tumefaciens*/GUS gene combinations, may be indicative of all callus explants being assayed at week 6 following inoculation. For all other *A. tumefaciens*/GUS gene combinations, a small percentage of explants were assayed at week 6 following inoculation, with the remaining explants assayed at week 15 following inoculation. If GUS expression was transient or toxic to carrot cells, callus explants remaining on culture medium until week 15 may have shown a decrease in GUS activity. More likely, callus cultures degraded over time and tissue death resulted in a decrease in or inability to detect GUS activity.

The LBA4404 strain of *A. tumefaciens* did not produce as many carrot transformants when compared to the AGL1 strain and the *A. tumefaciens* strains containing the GFP related gene constructs did not yield any regenerants. These results indicate some degree of incompatibility and/or toxicity on the carrot callus tissue by the strain of *A. tumefaciens*.

For both strains of *A. tumefaciens* there appeared to be early success in transformation of celery petioles for all the gene constructs used in this study (Table 5.5), albeit at very low levels. Regeneration of transgenic plants from celery callus was not achievable despite the use of different *A. tumefaciens* strains, binary plasmids and regeneration systems. Due to the consistency of these negative results from all the constructs tried it is likely that *A. tumefaciens*, or some of the reagents required in the transformation induction medium (ie. Acetosyringone) is toxic to the celery callus tissue.

The results from this study demonstrated that carrot cultivars are amenable to *A. tumefaciens*-mediated transformation, particularly carrot cultivar Crusader. While celery is a close relative to carrot, belonging to the same family Apiaceae, its response to *A. tumefaciens*-mediated transformation was less favourable. Tendercrisp was not amenable to transformation systems in these experiments and is consistent with earlier findings in this study (Chapter 3). The existence of only two reports of previous

celery transformation could be an indication that celery is not amenable to *A. tumefaciens*-mediated transformation (Catlin *et al*. 1988; Liu *et al*. 1992). Although both sources claim celery is amenable to *A. tumefaciens*-mediated transformation, neither investigations looked at the reporter gene GFP and only one source reported stable integration of a transgene into the celery genome (Liu *et al*. 1992). This source also reported difficulty regenerating viable transgenic plants from callus cultures (Liu *et al*. 1992).

To improve the success of these transformation regeneration systems, particularly for celery, improvements on the induction media, pre-culture treatments, donor plant age, explant type and transformation protocols should be tried as any one of these variables can have a profund influence on gene transfer. Alternately other transformation methods, such as particle bombardment should be tried in an attempt to avoid any further toxicity issues.

4.5 References

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5.0 Isolation, characterisation and incorporation of the NIa/NIb region from CarVY and CeMV into an ihpRNA construct.

5.1 Introduction

Conventional breeding for CarVY and CeMV resistance in carrot and celery has not been reported, necessitating the need for the production of virus resistant plants using alternative methods such as gene technology. One such technology harnesses the discovery that virus immunity via post-transcriptional gene silencing (PTGS) can be induced in plants that encode double-stranded (ds) or 'hairpin' (hp) RNA (Waterhouse *et al.* 1999; Smith *et al.* 2000; Wang and Waterhouse 2001). Researchers have developed a system to silence specific genetic sequence in planta, such as plant virus, by using anti-viral hpRNA cassettes that triggers transcriptional silencing of the plant virus (Wesley *et al.* 2001). The inclusion of a functional intron spacer region between the homologous arms of the hpRNA sequence increased the stability of the inverted repeat RNA by acting as an anchor or lynchpin (Wesley *et al.* 2001). Two generic ihpRNA vectors, pHannibal and pKannibal were subsequently designed, enabling the directional cloning of PCR products to form sense and anti-sense arms separated by a functional intron (Wesley *et al.* 2001). Commercial applications of ihpRNA-induced virus resistance that have used the pHannibal/pKannibal constructs to date include the development of barley plants showing immunity to Barley yellow dwarf virus (BYDV) (Wang *et al*. 2000; Abbott *et al.* 2001) and the generation of potato plants highly resistant to PVY (Isenegger *et al*. 2004). A similar ihpRNA vector based on the pHannibal/pKannibal constructs has also been used to generate PVY resistant potatoes (Missiou *et al.* 2004).

It has been proposed that the transgenes encoding dsRNA are more efficient than conventional sense or antisense viral transgenes in conferring virus resistance in plants (Waterhouse *et al*. 1998; Wang and Waterhouse 2001). Transgenic sequences that encode viral dsRNA trigger the PTGS system in plants to subsequently recognise invading virus and cleave the dsRNA into approximately 21-26 nucleotide fragments by the Dicer enzyme (Qi *et al*. 2002; Schauer *et al*. 2002; Papp *et al*. 2003; Tang *et al.* 2003). These small fragments, known as short-interfering RNA (siRNA), are then incorporated into the RISC complex and used as guides for cleavage of viral homologues on infection by virus (Hammond *et al*. 2000; Bernstein *et al*. 2001; Qi *et al*. 2002). While post-transcriptional silencing of viruses using anti-sense constructs typically results in only a small proportion of silenced individuals, intron-containing hairpin (ihp) RNA constructs can result in up to 100% of the independent transgenic plants showing silencing (Wesley *et al*. 2001).

The isolation and characterisation of genomic regions from CarVY and CeMV, and the incorporation of a suitable short viral sequence into intron-containing 'hairpin' (ihp) RNA constructs, would potentially enable the development of transgenic carrot and celery breeding lines that are resistant or highly tolerant to infection by CarVY and CeMV. Partial genomic sequence exists for three CarVY isolates (AF203538, AF203537, AF203539 and six CeMV isolates (AF203531, AF203532, AF203533, AF203534, AF203535, CM0271087) in the GenBank database. These accessions encompass sequence information of the coat protein (CP) region and the 3' end of the nuclear inclusion b (NIb) coding region for both potyviruses (Moran *et al.* 2002). To avoid intellectual property associated with the CP and NIb sequences for potyviruses

(pers. comm. P. Waterhouse), the NIa region of the CarVY and CeMV genome, which is located upstream of the CP and NIb, was targeted for incorporation into an ihpRNA resistance construct. A combination of reverse transcriptase - PCR (RT-PCR), using homologous and degenerate primers, and standard cloning strategies was used to determine the sequence of the NIb and NIa regions of the CarVY and CeMV genomes. This method has been successfully applied to identify novel sequences using degenerate primers based on closely related potyviruses (Langeveld *et al*. 1991; Colinet & Kummert 1993; Colinet *et al*. 1994; Gibbs & MacKenzie 1997).

The aims of this study were to: (1) isolate CarVY and CeMV from infected carrot and celery plants; (2) detect, sequence and characterise the NIa/NIb coding regions of CarVY and CeMV, and; (3) incorporate a suitable short region of CarVY and CeMV viral sequence into ihpRNA cassettes for subsequent transformation into plant tissue.

5.2 Materials and methods

5.2.1 Plant materials

Foliar and petiole tissue from carrot (*Daucus carota* var. *sativum*) and celery (*Apium graveolens* var. *dulce*) plants that were infected with Victorian isolates of CarVY and CeMV (AF203537 and AF203535 respectively) were provided by the Department of Primary Industries, Knoxfield, Australia.

5.2.2 RNA extraction

Viral RNA was extracted from carrot and celery petiole and leaf tissue using an adapted protocol derived from Alberts *et al*. (1989) with all steps carried out at 0-4ºC as follows: Homogenisation of infected tissue was done using extraction bags (Bioreba Reinach, Switzerland) and a HOMEX tissue homogeniser (Bioreba Reinach, Switzerland) in an extraction buffer consisting of 0.5 M sodium phosphate buffer, pH 7.0, containing 1 M urea, 0.5% thioglycolic acid (v/v) and 10mM sodium diethyl dithiocarbamate (1.5 mL of extraction buffer to 1 g of leaf tissue). The slurry was emulsified with chloroform (0.8 mL/mL slurry) for 1 min and centrifuged for 15 min at 7000g. The aqueous phase was removed and suspended in 0.25 M NaCl and 4% PEG (w/v) and stirred overnight at 4ºC prior to centrifugation for 15 min at 7000g. The pellet was resuspended in 1/5 the original volume of extraction buffer and Triton X100 was added to a final concentration of 1% (w/v). After stirring for 2 hrs, the preparation was spun (7000g) for 1-2 min and the aqueous phase collected. To 490µL of the resulting preparation, 500 µL of RNA extraction buffer (MacKenzie *et al*. 1997), 10 µL of β-Mercaptoethanol and 100 µL of 20% Sarkosyl was added and incubated in a water bath at 70ºC for 10 min. RNA extraction from the partially purified virus preparation was completed as described by the Qiagen RNeasy® Plant Minikit (Qiagen Pty Ltd, Australia) following the protocol for RNeasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi. Minor modifications were made to obtain high concentrations of total RNA from the partially purified virus preparations as follows: 4 x 450 µL aliquots of virus preparation were passed through the one QIAshredder spin column and 225 µL of 96-100% ethanol was added to each 450 µL aliquot. Eight 675 µL aliquots were applied to one RNeasy mini column and the remaining steps were carried out as specified in

the manufacturer's protocol. Total RNA was quantified using a GenQuant spectrophotometer (Amersham, Sweden).

5.2.3 Verification of virus presence

5.2.3.1. Immunological assays

Polyclonal antibodies to CarVY and CeMV were obtained from Deutsche SammLung von Mikroorganismen und Zellkulturen (DSMZ), GmbH, Germany (Catalogue Nos. AS-0148 and AS-0759, respectively). Leaf samples were extracted (1 g leaf/20 mL buffer) in phosphate buffered saline (0.14 M sodium chloride; 1.5 mM monobasic potassium phosphate; 6.7 mM dibasic sodium phosphate; 2.7 mM potassium chloride; 3 mM sodium azide), pH .4, containing 5% of Tween 20 and 2% of polyvinyl pyrrolidone. The extracts were tested in a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as described by Clarke and Adams (1977). Each test sample, together with positive (virus) and negative controls (no virus), were assayed in duplicate wells of a microtitre plate. Substrate buffer (0.92 M diethanolamine; 3 mM sodium azide; pH 9.8) was added to each well and incubated at room temperature for 1 hr. Absorbance values (A_{405nm}) were measured in a Multiscan plate reader (Labsystems, Finland) and samples with optical densities more than twice those of the corrected healthy control optical densities were considered to be positively infected.

5.2.3.2. Reverse transcriptase-polymerase chain reaction

The SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Life Technologies, USA) was used to amplify CarVY and CeMV cDNA transcribed from purified RNA for the purpose of virus identification. The constituents of the RT-PCR reaction were as follows: 12.5 µL X2 Reaction Mix; 1.0 µM each of primer 1 and 2; 0.5 µL SuperScript™ III Platinum® Taq DNA; 10.0 ng purified RNA; and the remaining volume with H_2O. RT-PCR reactions were carried out in a DNA Engine DYAD™ Thermocycler (MJ Research, USA) using the program depicted in Table 5.1. The broadly specific Potyvirid Primer 2 (PotyP2) (Gibbs & Mackenzie 1997) together with the CarVYP1 and CarVYP2 primers for carrot and the CeMVP1 and CeMVP2 primers for celery (Table 5.2) were used to amplify a 200 bp and 300 bp fragment respectively from the 3' end of the cistron encoding the NIb protein of both viruses.

Table 5.1. RT-PCR program for viral identification

Stage 1	Step	Temp (°C)		Time	No. of cycles/Stage
	1	48	for	45 sec	1
	2	94	for	2 min	-
Stage 2	1	94	for	45 sec	35
	2	58	for	45 sec	-
	3	72	for	2 min	-
Stage 3		72	for	10 min	1

5.2.4 Primers used for amplification of Viral genomic RNA, cloning and sequencing of PCR products

To amplify cDNA transcribed from single-stranded genomic RNA of the NIa/NIb region of CarVY and CeMV, a series of sequence specific primers for upstream amplification were designed using sequences of both viruses (Carrot: AF203538, AF203537, AF203539; Celery: AF203531, AF203532, AF203533, AF203534, AF203535, CM0271087 (GenBank)) (Table 5.2). Degenerate primers from the NIa region ("pNIa" primers) were designed based on highly conserved regions of closely related potyviruses (Carrot thin leaf virus: AF203530; Celery yellow mosaic virus: AY049717; Clover yellow vein virus: AF203536; Plum pox virus: PP0243957; Potato virus A: PVCGA; Potato virus Y: PVYAAA; Sweet potato feathery mottle virus: D86371; Tobacco etch virus: TEVCGHAT; Turnip mosaic virus: TMVCAPP (GenBank)) as reported in Moran *et al*. (2002) and Latham and Jones (2004). Primers were designed by aligning sequences using the Clustal W algorithm using the default parameters (Thompson *et al*. 1994) and Pretty Box consensus within the Australian National Genomic Information Service Bionavigator package (www.angis.org.au).

CarVY and CeMV specific forward and reverse primers were designed as sequence data from cloned virus specific amplicons were obtained (Table 5.2). These primers were used in combination with the pNIa primers to obtain NIa and NIb sequence data upstream of the known viral genomic sequence.

The putative CarVY and CeMV NIa/NIb amplicons were checked for size on a 1% agarose gel and DNA bands to be cloned and sequenced were excised from the gel using the QIAquick Gel Extraction kit according to the manufacturers' specifications (Qiagen, Australia). Fragment concentrations were estimated by visualisation on a 1% agarose gel stained with ethidium bromide under UV-light by comparing with known concentrations of size standards (1kb+ ladder 1 µg/µL (Sigma-Aldrich, USA)). Gel purified amplicons were ligated into the pGEM-T easy vector (Promega Corporation, USA) and transformed into *Escherishia coli* strain JM109 (Promega Corporation, USA) according to the manufacturers' instructions. White colonies were randomly selected from LB plates (refer Appendix I) containing 100 µg/mL ampicillin, 0.049 mM 5-bromo-4-chloro-3indolyl-β-D-galactosidase (X-Gal) and 4.2 µM isopropylthio-β-D-galactosidase (IPTG). Individual bacterial colonies were grown under selection in 3 mL LB containing 100 µg/mL ampicillin (Sigma-Aldrich, USA) overnight. Plasmids were isolated from the bacteria via the QIAprep spin miniprep kit method (Qiagen, Australia), and those containing inserts of the correct size were identified via PCR using the same primers used for the original fragment isolation (Table 5.2).

The nucleotide sequence of each cloned amplicon was determined via cycle sequencing from the SP6 and T7 sites within the pGEM-T easy vector (Promega Corporation, USA) using the PRISM BigDye Terminator chemistry, Version 3.1 (Applied Biosystems, USA). Each insert was sequenced from a minimum of three colonies in both the forward and reverse direction to obtain a consensus sequence using the 3730S capillary sequencer (Applied Biosystems, USA). The identity of NIa/NIb potyviral sequences were verified by BLAST analysis (Altschul *et al.* 1997) of the amplicon sequence against known potyviral sequences obtained from the

GenBank databases. Only sequences that scored an *E* value corresponding to 84% homology or greater were considered as potyviral sequence.

Table 5.2. List of PCR primers designed to amplify the NIa/NIb region of the CarVY and CeMV genomes.

Primer Name	Sequence[#]	BP	Tm (ºC)	GC* (%)
PNIa•	5'-TNAAYATGTATGGDTWYGAYCC-3'	22	55	38.7
PNIa2	5'-TTYTGGAARCAYTGGATY-3'	18	55.1	27.8
PNIa2a	5'-TTYTGGAARCAYTGGATYVRMAC-3'	23	60.4	26.1
PNIa2b	5'-TTCTGGAAGCACTGGATTGG-3'	20	64.8	45.0
PNIa3	5'-CAAYTDHWDGARTTYDVRAA-3'	20	51.5	10.0
PNIa4	5'-TGGAABAARHSVBWMRTSRC-3'	20	52.8	15.0
PNIa5	5'-AAYATGTATGGDTWYGWCC-3'	19	51.0	31.6
PNIa6	5'-GATCCTGAAGACTTCTCAGCAG-3'	22	62.0	50.0
PNIa7	5'-GATCCAGAAGACTTCTCAGCAG-3'	22	62.0	50.0
Poty2[+]	5'-ACCACAGGATCCGGYTGHCCRCTRTTVCC-3'	32	77.0	46.9
CeMVF1	5'-GGGAAAAAGAAAGAGTACTTCCAAG-3'	25	63.1	40.0
CeMVP1	5'-CGATCCCATTCTAGTATTGAG-3'	21	57.7	42.9
CeMVP2	5'-GGTGAATGTGTATTTGAGTCC-3'	21	58.3	42.9
CeMVP3	5'-TTCTCTGTAGGCCTGAGTTCTGCT-3'	24	66.4	50.0
CeMVP4	5'-CGGGAGCATGCGACGTCGG-3'	19	76.7	73.7
CeMVP5	5'-CGGTTGAATATGGCTTGTGC-3'	20	50	64.3
CarVYF1	5'-CACGTTATTTGAATTATATTTCC-3'	23	55.9	26.1
CarVYF2	5'-GCAGCTCCGGCCGCCATG-3'	18	76.9	77.8
CarVYP1	5'-CCTATGTGCTGGTTCACTCG-3'	20	63.3	55.0
CarVYP2	5'-GAGATCGCTCAATTTACCCG-3'	20	63.4	50.0
CarVYP3	5'-GTAACAAAACATGTGGTGAAAGG-3'	23	61.9	39.1
CarVYP4	5'-GGAAATATAATTCAAATAACGTG-3'	23	55.9	26.1

*GC% of non-degenerate bases. [#]B = GTC; D = GAT; H = ATC; S = GC; R = AG; V = ACG; W = AT; Y = CT. [+]Gibbs and Mackenzie (1997). • PNIa primer series, sense degenerate primers based on related potyviruses. CarVY and CeMV primer series, primers based on published or characterised sequence information (P-series sense; F-series anti-sense)

5.2.5 Construction of the ihpRNA constructs (pPOPOV-CarVY and pPOPOV-CeMV)

Standard gene cloning methods (Sambrook and Russell 2001) were used to generate ihpRNA constructs. The target viral region to be used for the ihpRNA construct was amplified from CarVY and CeMV RNA using the RT-PCR protocol defined in section 6.2.3 and the following primer sequences:

CarVY: 5'-GCGAAGCTTGGTACCCTAACTTAACTGGTTTAAACAATCC-3' and 5'-GCGTCTAGACTCGAGTTCTGGAAGCACTGGATTG-3';

CeMV: 5'- GCGTCTAGACTCGAGCCTATTTGAACACACATCCAG-3' and 5'-GCGAAGCTTGGTACCCAGTGTACTCTTGGAAGTAC -3'

The primers introduced *Xba*I/*Xho*I and *Kpn*I/*Hind*III restriction enzyme sites at the 5' and 3′ ends of the forward and reverse primers respectively (Figure 5.1). These assist the directional cloning of the PCR product into the pKannibal vector. The underlined nucleotides, in the above primers are the *Xba*I/*Xho*I and *Kpn*I/*Hind*III restriction enzyme sequences. The amplified CarVY and CeMV fragments, incorporating the restriction enzyme sites, were verified for size as described previously.

Purified fragments and the pKannibal vector (Kindly supplied by P. Waterhouse, CSIRO Plant Industry) (AJ311873 (GenBank)) were first digested with *XhoI* and *KpnI* and the fragment ligated into the corresponding multiple cloning sites of the pKannibal plasmid (Figure 5.1). Restriction enzymes (10 U/µL for *Xho*I, *Kpn*I and *Hind*III; 50 U/µL for *Xba*I) and corresponding reaction buffers were obtained from Invitrogen, Life Technologies, (USA). In the instance where reaction buffers for restriction enzymes differed, as occurred for *Xho*I and *Kpn*I, a double digest was carried out as follows: The enzyme requiring the lower concentration of salt, in this instance *Kpn*I, was used first to digest the target DNA (Sambrook 2001). Digestion of pKannibal and purified viral fragments was conducted for 1 hr at 37°C in a 20 µL reaction mix containing 12 µL water, 2 µL X10 React 4 buffer, 5 µL plasmid or viral fragment and 1 µL *Kpn*I restriction enzyme. The plasmid pKannibal and viral fragment were purified as follows: 2 µL sodium acetate (3M) was added to the 20 µL of reaction mix followed by 50 µL ethanol (96-100%) and the resulting 72 µL reaction was left on ice for 10 min. The reactions were spun at 15,000 g for 15 min and the resulting pellets were resuspended in 17 µL water. To complete the double digest, 2 µL of X10 React 2 buffer and 1 µL of *Xho* restriction enzyme were added to the resuspended pKannibal and viral fragment and incubated for 1 hr at 37°C. The presence and size of the double digested pKannibal plasmid and the virus-specific PCR fragment were verified prior to ligation by visualising under UV light a fraction of the digest that had been run on a 1% agarose gel and stained with ethidium bromide.

Ligation was carried out with a vector:fragment molar ratio of 1:7. Each 20 µL ligation reaction contained 10 µL 2X Rapid Ligation Buffer (Promega Corporation, USA), 7 µL double digested viral fragment, 1 µL double digested pKannibal vector and 2 µL T4 DNA Ligase (3 U/µL) (Promega Corporation, USA). Ligation reactions were incubated overnight at 4 °C. Ligated plasmids were transformed into *E. coli* strain JM109 (Promega Corporation, USA) according to the *E. coli* transformation protocol defined for the pGEM-T easy cloning vector (Promega Corporation, USA). Transformed *E. col*i cells, in solution, were plated on agar-solidified LB plates (150 µL/plate) containing 100 µg/mL kanamycin (Sigma-Aldrich, USA) and left at 37 °C overnight. Colonies were randomly selected from plates and grown overnight at 37 °C in 3 mL liquid LB with selection. Plasmids were isolated from the bacteria via the QIAprep spin miniprep kit method (Qiagen, Australia), those containing inserts of the correct size were determined by restriction with *Xho*I and *Kpn*I under reaction conditions as previously described.

The resulting pKannibal vector containing a sense viral insert was then digested, together with the original purified viral fragment containing the restriction enzyme sites, with *Xba*I and *Hind*III (Figure 5.1). In this instance restriction enzymes had a common reaction buffer, therefore double digests were carried out with both enzymes in one 30 µL reaction containing 8 µL water, 3 µL 10X React 2 buffer, 15 µL plasmid

or viral fragment and 2 µL each of *Xba*I and *Hind*III. The anti-sense viral fragment was cloned into the corresponding multiple cloning site of pKannibal already containing the sense viral insert as previously described.

pKannibal constructs containing both sense and anti-sense inserts of the correct size were determined by restriction enzyme digest with both *Xba*I/*Hind*III and XhoI/KpnI restriction enzymes as previously described. To further confirm that directional cloning was successful, a restriction enzyme digest was carried out using *Xba*I, *Hind*III and *Xho*I restriction enzymes. Each 20 µL reaction contained 9 µL water, 3 µL 10X React 2 buffer, 5 µL plasmid and 1 µL each of *Xba*I, *Hind*III and *Xho*I and was incubated for 1.5 hr at 37°C. The sizes of resulting fragments were verified by visualising under UV light on a 1% agarose gel prior to ligation.

The ihp cassette, together with the 35S constitutive promoter from the cauliflower mosaic virus (CaMV; Harpster *et al.* 1988) and the octopine synthase (*ocs*) terminator (MacDonald *et al*. 1991), were excised from the pKannibal vector with *Not*I and ligated into the T-DNA region of binary vector pArt27 containing an intron-interrupted kanamycin resistance gene (nos-*nptII*) as the selectable marker (Gleave 1992). The pKannibal vector containing a sense and antisense viral insert was digested, together with pArt27 using the following 32 µL reaction: 8 µL water, 3 µL 10X React 3 buffer, 15 µL plasmid and 2 µL *NotI* (15 U/µL). The ihpRNA cassette from pKannibal was cloned into the *NotI* cloning site of pArt27 as previously described.

Plasmids, referred to as pPOPOV-CarVY and pPOPOV-CeMV from this point onwards, were transformed into *E. coli* strain JM109 as previously defined. Colonies were randomly selected from LB plates containing 50 µg/mL spectinomycin (Sigma-Aldrich, USA) and grown overnight in 3 mL LB under the same antibiotic selection. Plasmids were isolated from cells using the QIAprep spin miniprep kit and protocol (Qiagen, Australia) and analysed for correct insert size by restriction enzyme digest using combinations of the following enzymes: *Xba*I, *Hind*III and *Xho*I as previously described.

Electrocompetent *Agrobacterium tumefaciens* AGL1 cells were prepared as follows: 2 mL YEP media containing 50 µg/mL spectinomycin (Sigma-Aldrich, USA) and 50 µg/mL rifampicillin (Sigma-Aldrich, USA) was inoculated with AGL1 cells (Lazo *et al*. 1991) and incubated at 28°C for 24 hr with shaking. The 2 mL pre-culture was added to 100 mL YEP media containing antibiotics in an autoclaved 250 mL flask and incubated at 28°C for 24 hr on a shaker until the OD_{600} was between 0.4 and 0.6. Cells were transferred to an autoclaved Oak Ridge tube (Nalge Nunc International, USA) and chilled on ice for 10 min before being spun at 10,000 rpm for 10 min at 4°C using a Avanti® J-E centrifuge (Beckman Coulter, USA). The supernatant was discarded following the spin and the pellet was resuspended in 20 mL ice cold 10% glycerol (Sigma-Aldrich, USA). The resuspended pellet was spun again at 10,000 rpm for 10 min at 4°C, the supernatant removed and the pellet suspended in 10 mL ice cold 10% glycerol. The previous step was repeated and the pellet resuspended in 1 mL ice cold 10% glycerol. Following a 3 min spin at 13,000 RPM (4°C), the resulting pellet was finally resuspended in 1 mL ice cold 10% glycerol. Aliquots (40 µL) of competent AGL1 cells were snap frozen in liquid nitrogen and stored at -70°C.

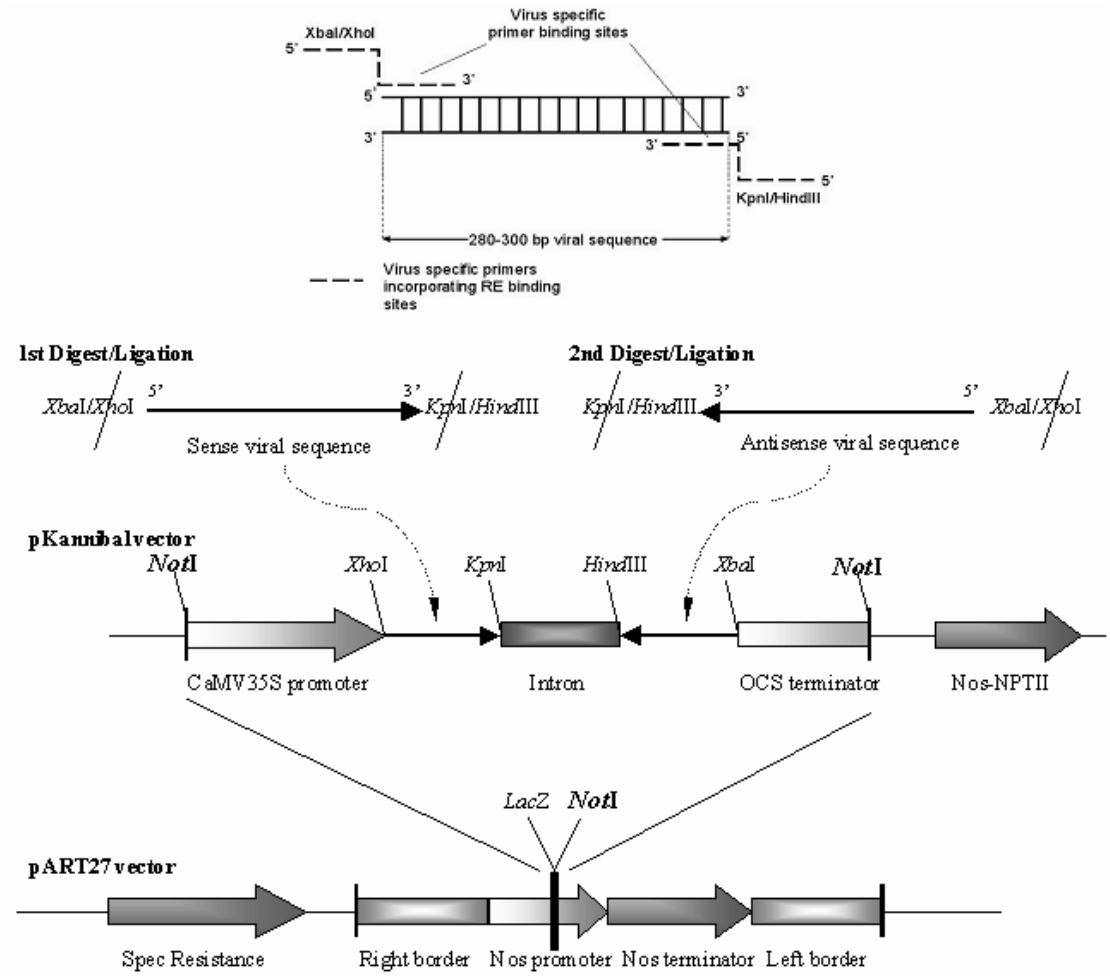

Figure 5.1. Construction of ihpRNA constructs targeted against CarVY and CeMV.

Plasmids, pPOPOV-CarVY and pPOPOV-CeMV, were transformed into the electrocompetent *A. tumefaciens* strain AGL1 by electroporation as follows: Five microlitres of pPOPOV-CarVY or pPOPOV-CeMV plasmid DNA (10 ng) was added to 40 µl of electrocompetent AGL1 cells and transferred to a pre-chilled 2 mm cuvette (EuroGentec, Belgium). Electroporation (1-2 sec pulse) was performed using an EasyjecT electroporation unit (EuroGentec, Belgium) with a voltage output of 2500 v. Following electroporation, cuvettes were placed on ice and 1 mL of sterile SOC medium was added. *A. tumefaciens* cells in SOC medium were incubated with shaking for 2-3 hrs at 28ºC. Cell suspensions were plated on YEP plates with 50 µg/mL spectinomycin and 50 µg/mL rifampicillin and incubated for 24-48 hr at 28ºC until colonies were visible. Single colonies were removed from selection plates and grown in 6 mL of liquid YEP medium with 50 µg/mL spectinomycin and 50 µg/mL rifampicillin for 24-48 hr at 28ºC.

A protocol modified from the QIAprep spin miniprep kit protocol (Qiagen, Australia) was used to isolate the plasmids from *A. tumefaciens* cells. Pelleted cells were resuspended in 250 µl of *A. tumefaciens* Plasmid Extraction Buffer in place of the P1 buffer supplied in the kit. The remaining steps were carried out as defined in the manufacturer's handbook (Qiagen, Australia). Plasmids, derived from *A. tumefaciens* cells, were analysed for correct insert size by multiple restriction enzyme digests (as carried out for *E. coli* plasmids and detailed in Section 5.2.5). For PCR confirmation

of antisense insert, when restriction enzyme digests yielded faint bands, the following primers targeting the virus-specific insert were designed based on regions within the pKannibal vector flanking the antisense orientation multiple cloning site:

Forward: pPOPOV-F: 5'-GAAATTGGGTTCGAAATCGAT-3'
Reverse: pPOPOV-R: 5'-CATATCTCATTAAAGCAGGAC-3'

The constituents of the PCR reaction were as follows: 2.5 µL X10 PCR buffer; 3mM $MgCl_2$; 0.24 mM dNTP mix 1 unit Taq DNA polymerase (Gibco-BRL, Life Technologies, USA); 0.4 µM each of primer pPOPOV-F and pPOPOV-R; 18.5 µL H_2O; and 10.0 ng purified plasmid DNA. PCR reactions were carried out in a DNA Engine DYAD™ Thermocycler (MJ Research, USA) using the program described in Table 5.3.

Table 5.3. PCR program for pPOPOV antisense insert verification.

Stage 1	Step	Temp (°C)		Time	No. of cycles/Stage
	1	94	for	1 min	1
Stage 2	1	94	for	10 sec	35
	2	55	for	30 sec	-
	3	72	for	1 min	-
Stage 3		72	for	5 min	1

To further confirm presence of the viral regions in the ihpRNA constructs, PCR derived viral fragments were excised from agarose gels, ligated back into the pGEM-T easy vector (Promega Corporation, USA), transformed into the *E. coli* strain JM109 (Promega Corporation, USA) and sequenced as previously described. The identities of the CarVY and CeMV sequences, derived from pPOPOV-CarVY and pPOPOV-CeMV respectively, were verified by Clustal W alignment using the default parameters (Thompson *et al*. 1994) with previously characterised sequences on the GenBank database as previously mentioned.

5.3 Results

5.3.1 Sequencing of CarVY NIa/NIb coding region

The region of the CarVY genome that encodes the previously unsequenced 5' end of the NIb protein and the 3' end of the NIa protein was amplified using the primer pair CarVYP2/PNIa2b (Figure 5.2). The nucleotide sequence of the degenerate primer PNIa2b is based on a conserved region found in closely related potyviruses, which is located approximately 1000 bp from the 5' end of NIa protein (Figure 5.3). The expected fragment size using the CarVYP2/PNIa2b primer combination was estimated to be approximately 1.6 kb and the actual fragment generated using the primers CarVYP2/PNIa2b was 1.432 kb (Figure 5.4). The CarVYP2/PNIa2b PCR product was cloned and sequenced and the 1389 bp consensus sequence (minus the primer sequences) was submitted to GenBank (Accession number: DQ174243). Analysis of the 1389 bp CarVY sequence using Frames (GCG) and Translate (GCG) programs (www.angis.org.au) identified a continuous open reading frame (ORF) and the highly conserved GDD motif was evident in the translated sequence (Figure 5.5). BLAST analysis confirmed >84% sequence homology to regions within a number of potyviruses including Bean yellow mosaic virus (BYU47033), Potato virus A

(AF543709), Lettuce mosaic virus (LMO306288), Tobacco etch virus (TEVCAPG), Papaya ringspot virus (AY162218), Lily mottle virus (TBR310203), Bean common mosaic virus (BCO012651), Plum pox virus (AF401296), Japanese yam mosaic virus (AB027007) and Cocksfoot streak virus (AF499738).

For further upstream amplification, a range of primer pair combinations was used to isolate the 3' end of the NIa protein (Figure 5.2). Although primer pairs targeting the 3' end of the CarVY NIa region yielded clear and reproducible single fragments, subsequent sequencing and analysis determined that these fragments were not of the target viral genome. Subsequently, sequence encoding the 3' end of the NIa protein (nucleotides 1-258 of Genbank Accession Number DQ174243) was used for incorporation in the ihpRNA constructs.

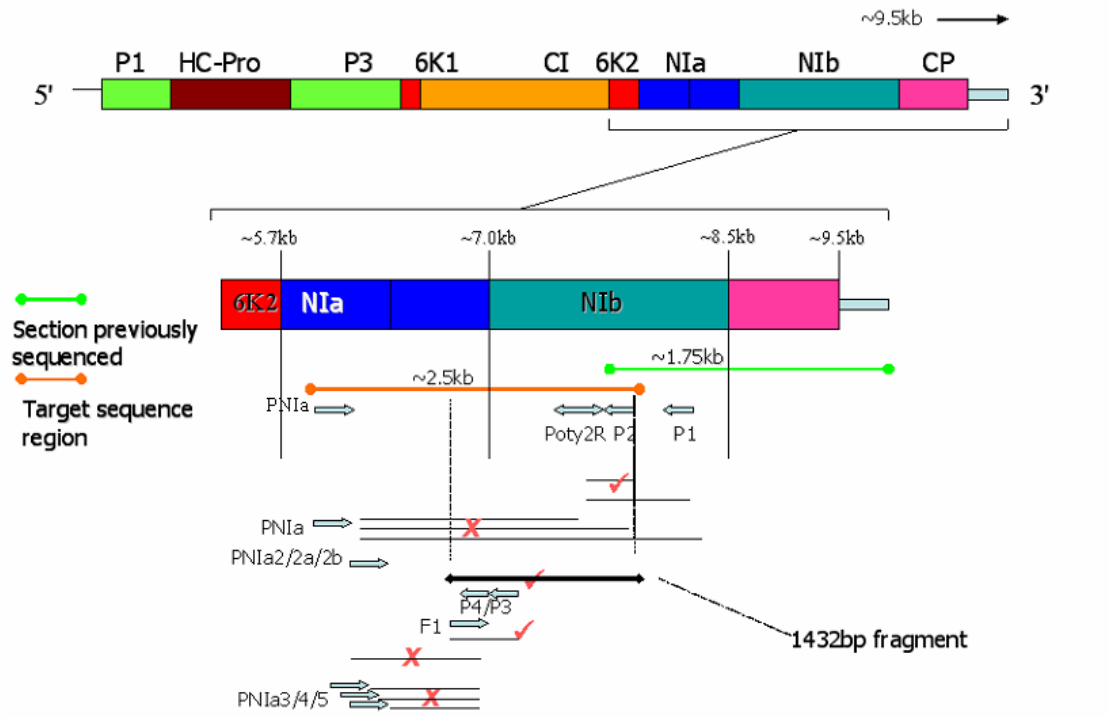

Figure 5.2. Isolation of the CarVY NIa/NIb potyvirus sequence. Ticks and crosses indicate successful and unsuccessful isolation attempts, respectively.

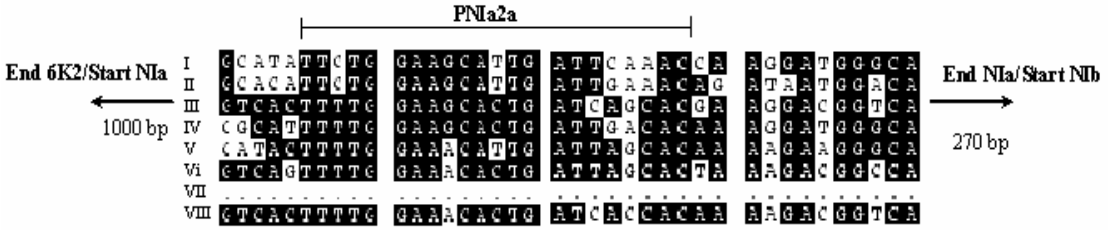

Figure 5.3. Position of PNIa2a degenerate primer based on a conserved region in closely related potyviruses. I. Tobacco etch virus - TEVCGHAT; II. Potato virus Y - PVYAAA; III. Plum pox virus – PP0243957; IV. Sweet potato feathery mottle virus – D86371; V. Potato virus A - PVCGA; VI. Turnip mosaic virus – TMVCAPP; VII. Celery yellow mosaic virus – AY049717; VIII. Japanese yam mosaic virus – AB016500.

Figure 5.4. 1432 bp nucleotide sequence of the 3' end of the NIa protein and the 5' end of the NIb protein from CarVY. The shaded area indicates the presumed NIa/NIb junction. Underlined bases indicate the PNIa2b binding site at the 5' end and the specific CarVYP2 binding site at the 3' end.

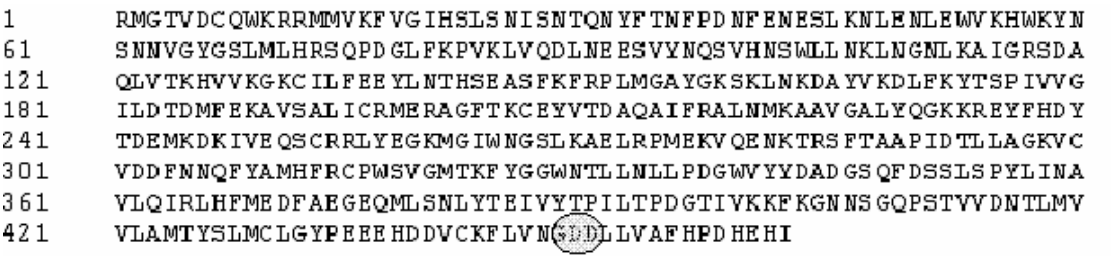

Figure 5.5. Amino acid (ORF) sequence of the 3' end of the NIa protein and the 5' end of the NIb protein from CarVY.

5.3.2 Sequencing of the CeMV NIa/NIb coding region.

The cDNA region encoding the previously unsequenced 5' end of the NIb protein of CeMV was successfully amplified using the primer pair CeMVP2/PNIa and characterised (Figure 5.6). The degenerate primer, PNIa, was based on a conserved region in the NIa protein in closely related potyviruses, approximately 180 bp from the 5' end of the NIa protein (Figure 5.7). The expected fragment size using primer pair CeMVP2/PNIa was estimated to be approximately 2.5 kb. However, the CeMVP2/PNIa PCR yielded a fragment of only 1050 bp (Figure 5.8) (DQ118375 (GenBank)). Sequence analysis showed that the downstream CeMVP2 primer non-specifically annealed to a site located in the 5' region of the NIb cistron and resulted in the generation of the observed amplicon (Figure 5.8). Primer sequence (including the non-specific CeMVP2 binding site at the 5' end of the NIb cistron) was deleted from the 1050 bp CeMV fragment before submission of the 1029 bp sequence to GenBank (Accession number DQ118375). Analysis of the 1029 bp CeMV virus fragment using Frames (GCG) and Translate (GCG) programs (www.angis.org.au)

identified a continuous ORF containing no Stop or Start codons and the highly conserved GDD motif that is located in the Nib coding regions of potyviruses (Figure 5.9). BLAST analysis confirmed >84% homology to regions of potyviruses; Beet mosaic virus (AY206394), Pepper mottle virus (AF501591), Soybean mosaic virus (S42280), Johnson grass mosaic virus (JGMVVPS), Bean common mosaic necrosis virus (BCU1927), Sweet potato feathery mottle virus (AF439637), Bean yellow mosaic virus (AY192568), Papaya ringspot virus (AY010722), Turnip mosaic virus (TMVNUCIN), Zucchini yellow mosaic virus (AF014811), Leak yellow strip virus (GPVPRC) and Tobacco vein mottling virus (POTTVMVX).

For further upstream amplification, a range of primer pair combinations was used to isolate the 3' end of the NIa protein (Figure 5.6). Another reverse primer, CeMVP5 (Table 5.2), was designed to avoid the non-specific CeMVP2 binding site within the viral fragment. Although the primer pairs that were targeted towards the 3' end of the NIa region yielded single, clear and reproducible fragments, subsequent sequencing and analysis determined that these amplicons were non-specific. As a result, the sequence encoding the 5' end of the CeMV NIb protein (nucleotides 1-313; DQ118375) was used for incorporation into the ihpRNA construct.

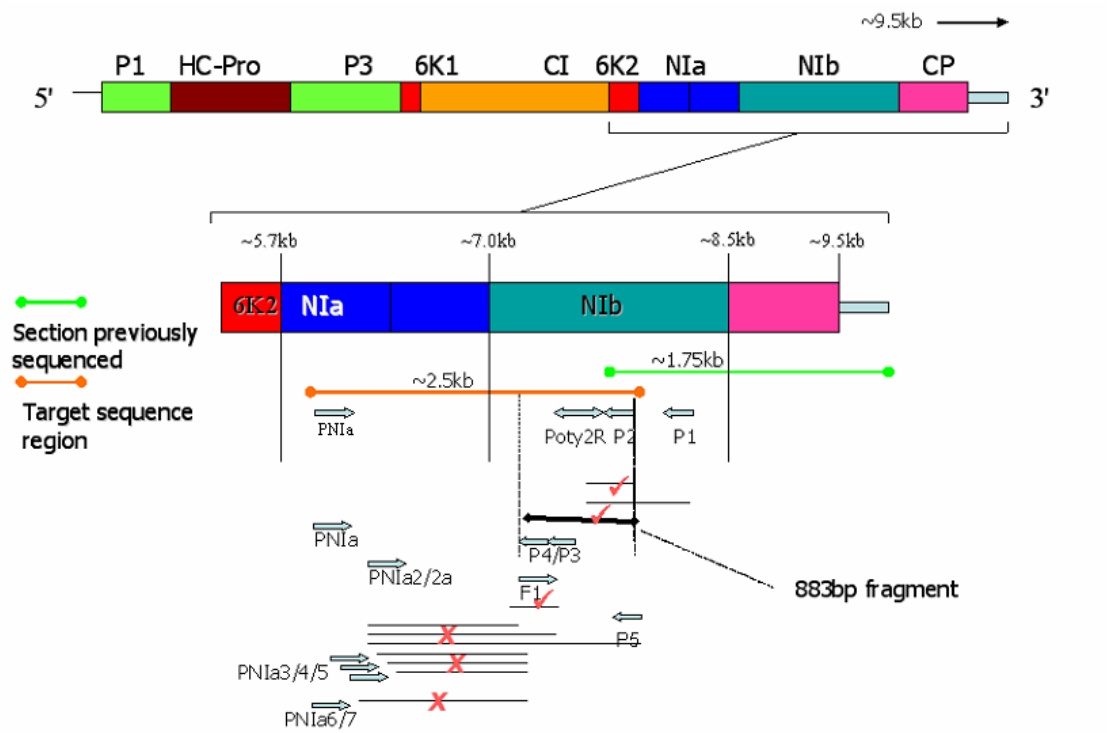

Figure 5.6. Isolation of the CeMV NIa/NIb potyvirus amino acid sequence. Ticks and crosses indicate successful and unsuccessful isolation attempts, respectively.

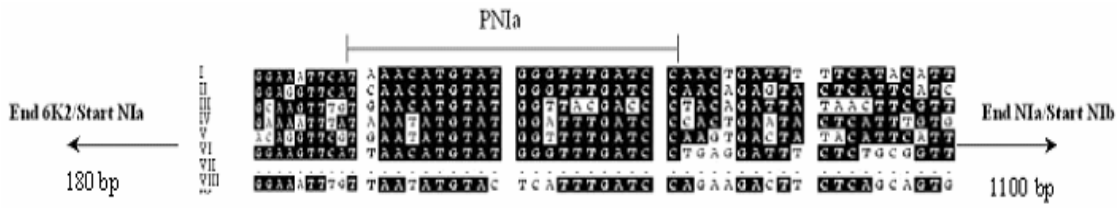

Figure 5.7. Position of the PNIa degenerate primer based on a conserved region in closely related potyviruses. I. Tobacco etch virus - TEVCGHAT; II. Potato virus Y - PVYAAA; III. Plum pox virus – PP0243957; IV. Sweet potato feathery mottle virus – D86371; V. Potato virus A - PVCGA; VI. Turnip mosaic virus – TMVCAPP; VII. Celery yellow mosaic virus – AY049717; VIII. Japanese yam mosaic virus – AB016500.

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   1   GGTGAATGTGTATTTGAGTCCTATTTGAACACACATCCAGAGGCAAACAACTTCTTTAGA
  61   CCATTCATGGGGGCGTATGGAAAGAGTAAATTGAATAAGGAAGCATACGTGAAGGACTTA
 121   TTCAAGTACACAAGCCCTATAGTCGTTGGAGTGCTTAACACGAACGTCTTTGAGCAGGCA
 181   GTTGAGTCAGTAATTAAGAGGATGGAAAAGTCAGGCTTTGATAAGTGTGAATATGTAACT
 241   GATGCACAAGCCATATTCAAGGCATTGAACATGAAAGCAGCTGTAGGAGCCCTTTACCAA
 301   GGGAAAAAGAAAGAGTACTTCCAAGAGTACACTGAAGAAATGCAAGATGAAATAGTCAAA
 361   CAGAGCTGTGAAAGACTATATAAAGGTAAAATGGGCATTTGGAATGGTTCATTGAAAGCA
 421   GAGCTCAGGCCTACAGAGAAAATTCAAGAGAACAAGACGAGATCATTTACAGCAGCGCCA
 481   ATAGACGCTTTATTGGCCGGAAAAGTTTGTGTGGATGATTTTAACAACCAGTTCTATGCT
 541   TCACATTTCAAGTGTCCGTGGAGTGTTGGAATGACAAAATTTTATGGGGGTTGGGATAAG
 601   CTCTTATCGCCCTTGCCTGATGGATGGGTTTACTATGATGCTGATGGTAGTCAATTCGAC
 661   AGTTCTTTGTCACCATACCTCATCAATGCCGTTTTGCAAATTCGGTTGCATTTCATGGAG
 721   GATTTTGACATTGGTGAGAAGATGCTATCAAATTTGTACACAGAAATAGTCTACACACCC
 781   ATTCTCACGCCAGATGGAACGATTGTGAAGAAGTTTAAGGGGAACAACAGTGGGCAACCA
 841   TCAACAGTAGTGGACAATACTTTGATGGTGGTTTTGGCAATGACGTACTCGTTAACGCTC
 901   CTTGGTTATGAAGAAAGTATACACGATGACGTGTGCAGATTCTTAATAAATGGGGACGAC
 961   TTACTTGTAGCATTTCATCCAGACCATGAACACATAGCAAGCAAGTTAGAAGACATCTTC
1021   AGAGAGATGGGACTCAAATACACATGCACC
```

Figure 5.8. The nucleic acid sequence data from the 5' end of the NIb protein from CeMV. Underlined bases indicate the non-specific CeMVP2 binding site at the 5' end and the specific CeMVP2 binding site at the 3' end.

```
1     GECVFESYLNTHPEANNFFRPFMGAYGKSKLNKEAYVKDLFKYTSPIVVGVLNTNVFEQA
61    VESVIKRMEKSGFDKCEYVTDAQAIFKALNMKAAVGALYQGKKKEYFQEYTEEMQDEIVK
121   QSCERLYKGKMGIWNGSLKAELRPTEKIQENKTRSFTAAPIDALLAGKVCVDDFNNQFYA
181   SHFKCPWSVGMTKFYGGWDKLLSPLPDGWVYYDADGSQFDSSLSPYLINAVLQIRLHFME
241   DFDIGEKMLSNLYTEIVYTPILTPDGTIVKKFKGNNSGQPSTVVDNTLMVVLAMTYSLTL
301   LGYEESIHDDVCRFLIMGDDLLVAFHPDHEHIASKLEDIFREMGLKYTCT
```

Figure 5.9. The amino acid (ORF) sequence of the 5' end of the NIb protein from CeMV.

5.3.3. Construction of pPOPOV-CarVY/CeMV ihpRNA vectors

The pPOPOV- vectors were successfully constructed and subsequently used for plant transformation to impart resistance to CarVY and CeMV (Figure 5.10).

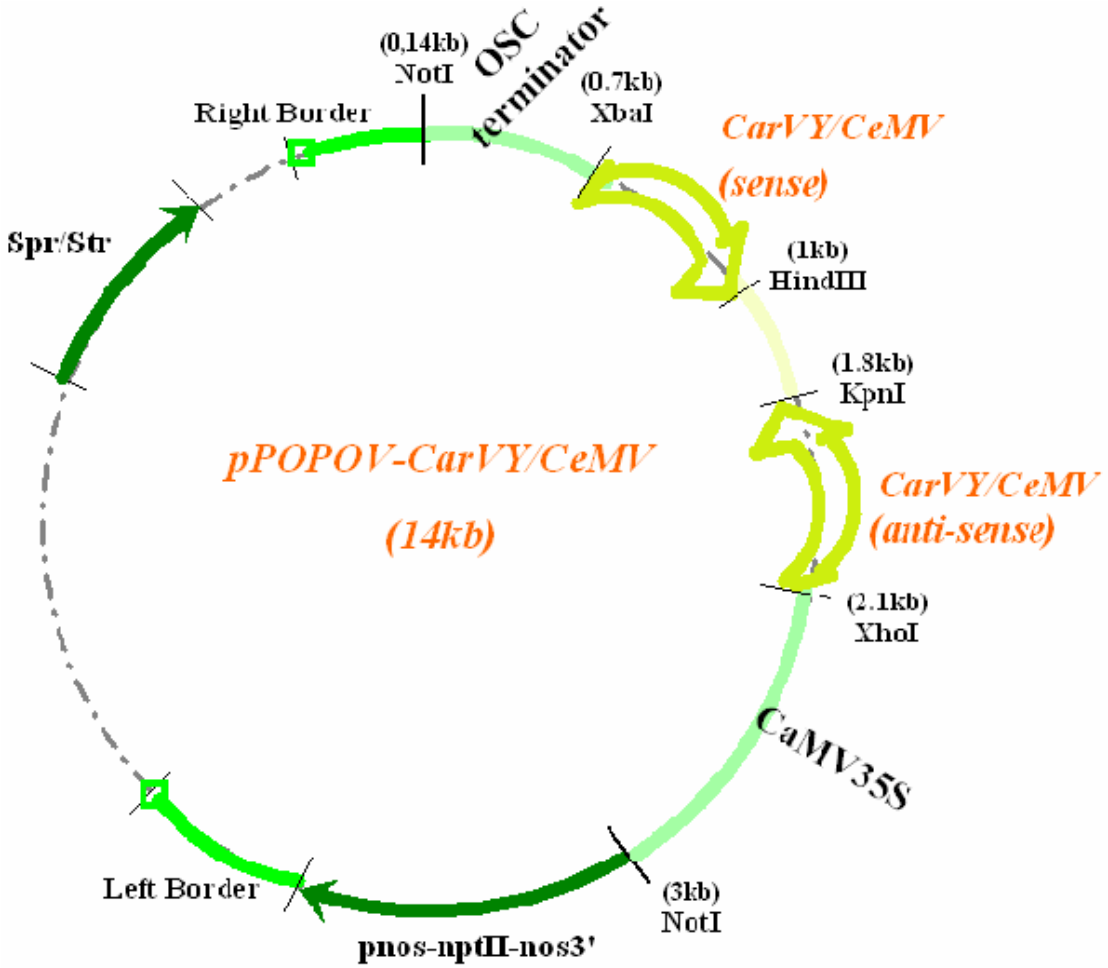

Figure 5.10. pPOPOV construct indicating the positions of the sense and anti-sense viral inserts, the CaMV35S promoter, the OSC terminator, left and right borders and the kanamycin resistance gene (*pnos-nptII-nos3'*).

Restriction enzyme digests and PCR analysis of pPOPOV-CarVY and pPOPOV-CeMV constructs for determination of correct insert size and position yielded fragments of the correct sizes (Table 5.4; Figure 5.11). The fragment generated by the *Xba*I/*Hind*III cut showed the presence of the sense orientation virus sequence and the *Hind*III/*Xho*I fragment showed the presence of the intron and anti-sense orientation viral sequence. The fragment generated by the *Xho*I/*Xba*I cut confirmed the presence of the remaining pKannibal and pART27 vector sequences. Primers targeting the antisense virus insert yielded the expected 305 bp and 370 bp fragments indicative of the antisense viral insert for pPOPOV-CarVY and pPOPOV-CeMV, respectively.

Table 5.4. Predicted fragment sizes for restriction enzyme and PCR checks on the pPOPOV-CarVY/CeMV construct.

Plasmid		Restriction Enzyme Digest	PCR	Expected and visualised fragment size(s)
pKannibal (control)		*Xba*I/*Hind*II/*Xho*I	-	<50bp (not visible); 800bp; 5.2kb
pKannibal (control)		-	PPOPOV specific primers	No fragment
PPOPOV ex. JM109	CarVY	*Xba*I/*Hind*II/*Xho*I	-	305bp; 1.1kb; 12.6kb
	CeMV	*Xba*I/*Hind*II/*Xho*I	-	370bp; 1.1kb; 12.6kb
PPOPOV ex. AGL1	CarVY	*Xba*I/*Hind*II/*Xho*I	-	305bp; 1.1kb; 12.6kb
	CeMV	*Xba*I/*Hind*II/*Xho*I	-	370bp;1.12kb; 12.6kb
PPOPOV ex. AGL1	CarVY	-	PPOPOV specific Primers	305bp
	CeMV	-		370bp

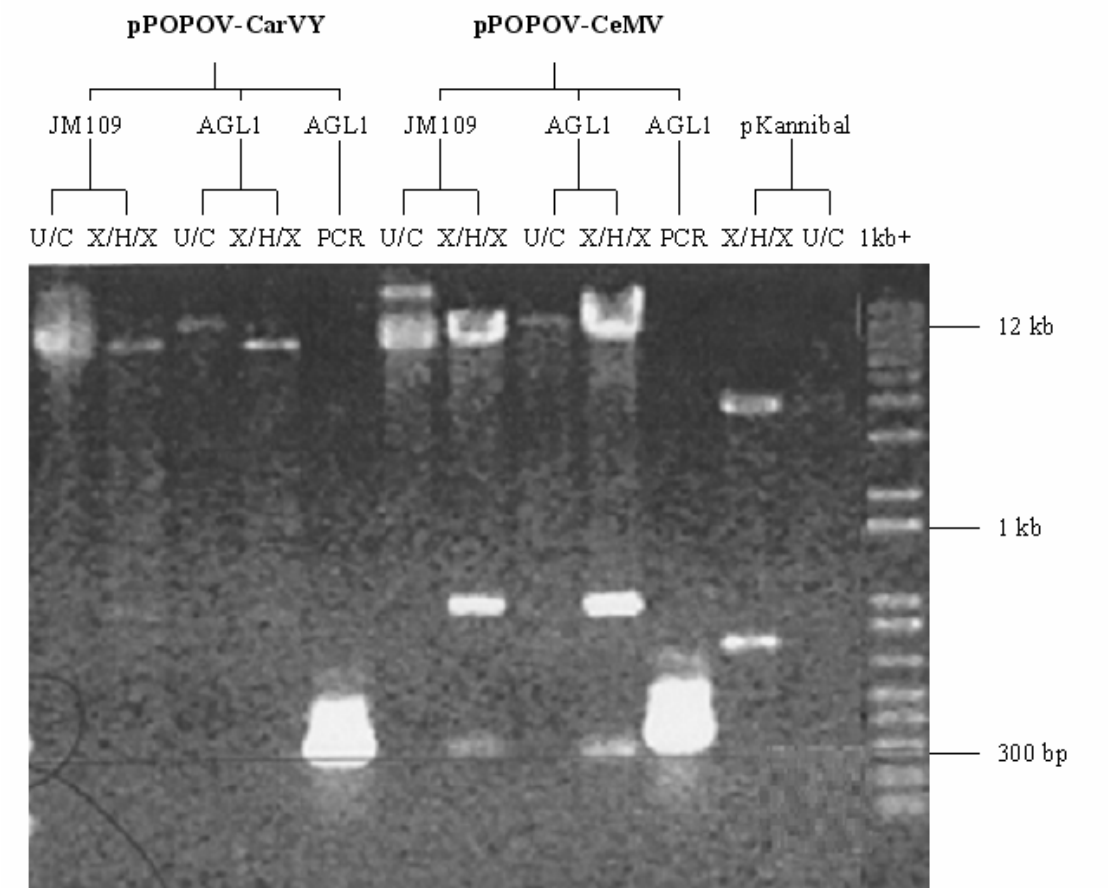

Figure 5.11. Restriction enzyme digest and PCR analysis of the pPOPOV-CarVY and pPOPOV-CeMV constructs for correctly sized inserts. Where U/C = uncut plasmid; XHX = *Xba*I/*Hind*III/*Xho*I digest. Note: XHX digest fragments for pPOPOV-CarVY ex. JM109 and AGL1 were faint, but clearly visualised under UV light.

5.4 Discussion

As an initial step towards the production of transgenic virus resistant carrot and celery genotypes, the genomic RNA of the single-stranded positive sense RNA viruses CarVY and CeMV were isolated and concentrated in the form of a partially purified virus preparation from infected carrot and celery plants using the protocol of Alberts *et al*. (1989). RNA was extracted from the partially purified virus preparations using RNeasy mini columns and viral RNA amplified using RT-PCR. The Potyvirid primer 2 (Gibbs and MacKenzie 1997), which was based on the conserved motif that encoded the amino acid sequence -GNN-, together with virus specific primers were successfully applied to further confirm the presence of CarVY and CeMV genomic RNA in the RNA extracts. Although degenerate primers have been applied in the past to detect members of the potyvirus family (Gibbs and MacKenzie 1997; Chen *et al.* 2001), sequence data of the target virus genome is required to definitively identify an individual virus.

Following RNA extraction and verification, the partial NIa/NIb coding regions of CarVY and the partial NIb coding region of CeMV were cloned and sequenced. In this study degenerate primers were successfully used to isolate the NIa and NIb sequences from CarVY and CeMV. Sequence alignment of closely related potyviruses showed high sequence homology in a number of areas within the NIa coding region enabling the design and successful implementation of degenerate primers for isolation of the NIa and NIb sequences from the CarVY and CeMV. Conserved amino acid sequences of the virion proteins, reported in potyvirus members, have previously been targeted for the design of degenerate primers to amplify cDNA of unknown viral sequences (Langeveld *et al*. 1991; Colinet and Kummert 1993; Colinet *et al*. 1994; Gibbs and MacKenzie 1997; Chen *et al*. 2001).

In the case of CarVY, the remaining unsequenced 5' portion of the NIb coding region and a 240 bp section of the 3' end of the NIa coding region was successfully cloned and sequenced. This enabled the use of CarVY NIa sequence for incorporation into ihpRNA constructs. Amplification of the NIa region of CeMV proved more difficult. While the majority of the remaining unsequenced 5' portion of the NIb coding region was successfully isolated and characterised, further upstream amplification into the NIa region was not achieved. This was potentially due to the presence of a strong secondary structure in the CeMV single-stranded RNA in the NIa/NIb region that may have prevented cDNA synthesis beyond a particular point. Partial NIb sequence has been previously used by Wang *et al.* (2000) and Isenegger *et al.* (2004) to design silencing cassettes for Barley yellow dwarf virus (BYDV) and Potato virus Y (PVY) respectively. Although to date there are no reports of the NIa region being used in ihpRNA constructs to target viral pathogens, NIa sequences of potyviruses have been successfully applied for other examples of pathogen-derived resistance (Maiti *et al*. 1993; Maiti *et al*. 1999).

For protection against CarVY and CeMV a 258 bp region of the CarVY NIa cistron (Genbank Accession number DQ174243) and a 313 bp region of the CeMV Nib cistron (Genbank Accession Number DQ118375) were each cloned into the pKannibal plasmid in a sense and anti-sense orientation. The reported size of viral fragments inserted into ihpRNA vectors such as pKannibal to successfully confer PTGS varies from between 197 bp to 1.6 kb (Wang *et al*. 2000; Kalantidis *et al*. 2002;

Pandolfini *et al.* 2003; Isenegger *et al.* 2004; Missiou *et al*. 2004). Wesley *et al*. (2001) reported that viral inserts as small as 98 bp can initiate efficient silencing. The use of smaller viral fragments in silencing vectors reduces the risk of viral recombination within transgenic plants. Transcription of the antiviral cassette in transformed tissues is predicted to trigger the post transcriptional gene silencing mechanism in plants and subsequently, invading virus particles are prevented from accumulating in plant cells as the dsRNA replicative form of RNA viruses is degraded (Waterhouse *et al.* 1998). This technology has been applied successfully for the development of barley plants immune to BYDV (Wang *et al*. 2000; Abbott 2001), tobacco showing complete resistance to Cucumber mosaic virus (Kalantidis *et al.* 2002), Plum pox virus (Pandolfini *et al*. 2003) and potato plants highly resistant to Potato virus Y (Isenegger *et al*. 2004; Missiou *et al.* 2004).

The formation of anti-viral ihpRNA cassettes targeted towards the recognition and/or transcriptional silencing of CarVY and CeMV will enable the development of carrot and celery breeding lines that are highly tolerant or resistant to infection by CarVY and CeMV. Transgenic carrot and celery plants will be produced containing integration of anti-viral ihpRNA cassettes into plant tissue via *A. tumefaciens*–mediated transformation and subsequent regeneration via somatic embryos (Chapter 6).

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6.0 The transformation and regeneration of carrot and celery with *Carrot virus Y* and *Celery mosaic virus* resistance constructs and the assessment of transgenic lines for virus resistance

6.1. Introduction

Carrot virus Y (CarVY) causes a devastating disease in the major carrot growing regions of Australia (Moran *et al*. 1999, 2002; Latham and Jones 2000, 2004), while Celery mosaic virus (CeMV) severely impacts celery growing regions worldwide (Severin and Freitag 1938; Chod 1984; Brandes and Luisoni 1966; Fry and Proctor 1968; Marchoux *et al*. 1969; Iwaki and Komuro 1970; Walkey *et al*. 1970; Avgelis and Quacquarelli 1972; Kemp and Froud 1975; Gracia and Feldman 1977; Alberts *et al.* 1989). Symptoms of CarVY and CeMV disease include a chlorotic mottle of the leaves, distortion of foliage and stunted plant growth (Carrot: Hay 2002; Latham and Jones 2004; Celery: Alberts *et al.* 1989; D'Antonio *et al.* 2001; Traicevski *et al.* 2001), the impacts of which result in crop losses. Both viral diseases are now present in all carrot and celery growing regions of Australia and although infection incidences vary greatly, infection levels can reach up to 90% causing major financial losses (Latham and Jones 2001; Traicevski *et al*. 2001).

The most effective measure for controlling CarVY and CeMV outbreaks is the instigation of a 'carrot/celery-free' period (Brunt *et al*. 1996; Latham and Jones 2000, 2003, 2004; Latham 2001; Latham *et al*. 2004). Although extension campaigns to maximise adoption of an integrated disease management strategy for CarVY and CeMV are currently under way in Australia (Latham and Jones 2004), breaking carrot production cycles is a drastic and costly option that growers are unlikely to adopt necessitating the need for alternative management strategies (Latham and Jones 2004; Latham *et al*. 2004). One such alternative, as recommended by industry, is the introduction of CarVY and CeMV resistant genotypes into breeding programs (Traicevski 2001). Sources of natural resistance to CarVY and CeMV are rare. Recently a resistant source was found for CeMV in California (D'Antonio *et al*. 2001) and to date there are no known sources of resistance for CarVY. The development of resistance sources of CarVY and CeMV via genetic engineering offers a realistic approach for the protection of valuable carrot and celery crops against diseases associated with these viruses.

Virus resistance, as a result of post transcriptional gene silencing, can be induced in plants by the introduction of partial RNA viral sequence into the plant genome in a double-stranded form (Waterhouse *et al*. 1998; Wang *et al.* 2000). Transgenes encoding virus-derived dsRNA are proposed to be more efficient than conventional sense or antisense viral transgenes in conferring virus resistance in plants (Waterhouse *et al*. 1998; Wang and Waterhouse 2001) and the inclusion of an intron in the hpRNA construct can increase the silencing effect to between 90 and 100% (Wesley *et al*. 2001). Generic ihpRNA vectors (ie pKannibal, see Chapter 5) enable directional cloning of PCR products to form sense and anti-sense arms that are separated by a functional intron (Wesley *et al.* 2001). An ihpRNA-induced virus resistance strategy has been demonstrated for the luteovirus, Barley yellow dwarf virus (BYDV) in barley (Wang *et al*. 2000; Abbott *et al.* 2001) and the generation of potato plants highly resistant to potyvirus Potato virus Y (PVY) (Isenegger *et al*. 2004; Missiou *et al*. 2004). In 2000, Wang *et al*. showed that 9 barley plants (from

25 independent barley lines transformed) carrying a 1.6 kb sequence from the 5' end of the Barley yellow dwarf virus (BYDV) polymerase NIb cistron were immune to BYDV. Isenegger *et al*. (2004) produced three transgenic potato lines expressing resistance to PVY infection by incorporating a 300 bp region of the NIb cistron into the plant genome.

The aims of this study were to:
introduce the pPOPOV-CarVY and pPOPOV-CeMV ihpRNA virus resistance constructs (generated in Chapter 5) into carrot and celery cells via *A. tumefaciens*-mediated transfer (using optimised protocols developed in Chapter 4),
selectively regenerate transformed cells into mature plants in the glasshouse, and;
challenge transgenic carrot and celery lines by inoculation with CarVY and CeMV respectively in glasshouse trials and determine the extent of virus resistance by ELISA.

6.2 Materials and Methods

6.2.1 Plant material

Four-week-old petiole material of celery (*Apium graveolens* var. *dulce*) cultivars Summit and Tendercrisp and carrot (*Daucus carota* var. *sativum*) cultivars Crusader and Stefano was used as a source of explant material for transformation experiments. Petiole material, derived from stock plant cultures, was maintained on agar-solidified, hormone-free MS salts and vitamins (Murashige and Skoog 1962) at 25ºC with a 16h/8h light/dark photoperiod. The petiole material was cut into 1 cm sections immediately prior to immersion in *Agrobacterium tumefaciens* cultures.

6.2.2 Bacterial strains and vectors

The *A. tumefaciens* strain AGL1 (Lazo *et al*. 1998), harbouring the plasmid pPOPOV-CarVY or pPOPOV-CeMV, was used for co-cultivation with petiole explants (refer to Section 5.2.5 for plasmid construction details). Engineered *A. tumefaciens* strains were maintained on selection plates of agar-solidified YEP medium containing 50 µg/mL spectinomycin and 50 µg/mL rifampicillin. Plates were stored at 4ºC and subcultured to a fresh plate monthly.

6.2.3 Growth and induction of *Agrobacterium tumefaciens* transformed with pPOPOV-CarVY and pPOPOV-CeMV

A. tumefaciens cultures used for transformation were induced by acetosyringone before co-cultivation with plant tissues. Two to three loops of actively growing *A. tumefaciens* (AGL1) colony was grown in 20 ml YEP medium containing 50 µg/mL spectinomycin and 50 µg/mL rifampicillin for 24 h at 28ºC in the dark with rotary agitation (200 rpm). *A. tumefaciens* cultures were centrifuged for 5 min at 5000 rpm, washed with sterile 0.9% NaCl, centrifuged and the resultant pellet resuspended in 100 mL of induction medium (0.5% glucose; 1 x AB salts; 30 mM MES (4-morpholineethanesulfonic acid); 2 mM sodium phosphate and 100 µM acetosyringone; pH 5.6). Following incubation for 16-18 h at 28ºC in the dark with rotary agitation (100 rpm), *A. tumefaciens* cultures were applied to the carrot and celery petiole explant material.

6.2.4 Plant transformation and regeneration

For the regeneration of transformed plants from carrot and celery petiole tissue, optimal media constituents were used as defined in Sections 2.2.3 and 3.2.3 respectively. Freshly prepared petiole explant material was placed in petri dishes and immersed for 10 min in a suspension of *A. tumefaciens* AGL1 harbouring pPOPOV-CarVY or pPOPOV-CeMV. The petioles were blotted briefly with sterile napkins to remove excess liquid, placed on co-cultivation basal medium comprising agar solidified MS for carrot tissue and B5 (Gamborg *et al*. 1968) salts and vitamins for celery tissue, and incubated in the dark at 22°C for 3 days. The petioles were then transferred to callus induction medium, basal medium augmented with 150 mg/L Timentin and containing 0.5 µM 2,4-D for carrot cultures and 100 mg/L serine, 2.5 µM 2,4-D and 3.0 µM kinetin for celery cultures. Petiole cultures were incubated for 4 weeks in the dark on callus induction medium prior to removal of callus protrusions. Callus explants were transferred to callus maintenance and proliferation medium; basal medium containing 0.5 µM 2,4-D, 150 µg/mL timentin and 25 µg/mL kanamycin for both carrot and celery callus cultures. Callus cultures were subcultured after 4 weeks, and the kanamycin concentration was increased to 50 mg/L to select for kanamycin-resistant calli which were subsequently transferred to PGR-free basal medium and a 16h/8h light/dark photoperiod for somatic embryogenesis and regeneration. Shoots with defined cotyledons and root structures were removed from callus cultures and placed on agar solidified MS containing 150 mg/L Timentin and 50 mg/L kanamycin and maintained at 25ºC with a 16/8 light/dark photoperiod for further development and maintenance. For asexual proliferation of transgenic plant lines, plants were placed on agar solidified MS containing 5 µM 6-benzylaminopurine (BAP), 150 µg/mL timentin and 50 µg/mL kanamycin and maintained at 25ºC with a 16h/8h light/dark photoperiod. Transgenic plants were routinely subcultured every 8 weeks.

6.2.5 Acclimatisation of tissue culture plants in the glasshouse

Transgenic plants were moved from *in vitro* conditions to a PC2 glasshouse for virus challenge trials. Well developed plants with comprehensive root systems and multiple petioles were washed with warm water (35–40 ºC) to remove traces of agar solidified growth medium. It was important to remove as much agar as possible from around the roots to ensure sugars contained in the medium did not promote the growth of pathogenic organisms in the soil-based medium. If basal callusing was excessive, callus protrusions were removed with a scalpel (being careful to maintain the integrity of the meristematic material) and plants were dipped in indole-3-butyric acid 800ppm in talc (Kendon, Australia). Plants were placed in 15 cm plastic pots containing autoclaved pine bark medium and vermiculite in a 50:50 ratio. To maintain high humidity and gradually acclimatise plants to new conditions, plastic cups were placed over the plants. Plants were irrigated using a drip irrigation system. Four weeks following the movement of plants to the glasshouse, plastic cups were removed and the plants were fertilised with Aquasol™ (Hortico Pty Ltd, Australia), using half the recommended application rate as indicated on the package (g/mL water). Plants were subsequently fertilised fortnightly.

6.2.6 Analysis of transgenic plants

6.2.6.1 Plant inoculations to test for Carrot virus Y resistance

Carrot leaf tissue infected with a Victorian isolate of CarVY (AF203537) was used to mechanically inoculate transgenic carrot plants of cultivar Crusader containing the pPOPOV-CarVY transgene that had been acclimatised in the glasshouse for 6 weeks. For mechanical inoculation of CarVY infected sap onto the test plants, 1 g of CarVY infected tissue (as determined by ELISA described in Section 6.2.6.4) was homogenised in 10 mL phosphate buffer containing 0.1% sodium sulphate using a clean mortar and pestle. As a negative control, transgenic and non-transgenic plants were inoculated with just phosphate buffer containing 0.1% sodium sulphate. Carborundum powder was sprinkled over the apical leaves (2-4) of each test plant and the sap inoculum was gently rubbed onto leaf surface to facilitate virus transmission. Following inoculation, the leaves were gently washed with sterile distilled water to remove traces of sap inoculum and carborundum powder.
For each transgenic line and non-transgenic control, 5 plants were treated with CarVY sap inoculum and 5 plants treated with phosphate buffer containing 0.1% sodium sulphate for comparison.

6.2.6.2 PCR

To identify transgenic plant lines that contain the pPOPOV-CarVY and pPOPOV-CeMV hairpin constructs, PCR analysis using the oligonucleotide primers pPOPOV-F (5'-GAA ATT GGG TTC GAA ATC GAT-3') and pPOPOV-R (pPOPOV-R: 5'-CAT ATC TCA TTA AAG CAG GAC-3') were used to amplify a 305 bp and 370 bp fragment from the sense arms of pPOPOV-CarVY and pPOPOV-CeMV respectively. For further confirmation of transgene integration, oligonucleotide primers pPOPOV-intron F (5'-GAC AAG TGA TGT GTA AGA CG-3') and pPOPOV-intron R (5'-GCA GAT TGG AAT TTC TAA C-3') were used to amplify a region of approximately 300 bp which forms part of the hairpin loop between sense and antisense sequences of the pPOPOV-CarVY and pPOPOV-CeMV construct. PCR analysis was performed according to the protocol specified in Section 5.2.5 (Table 5.3). Annealing temperatures for PCR reactions were 55 ºC and 56 ºC for antisense insert and the hairpin loop respectively.

6.2.6.3 Immunological assays

Polyclonal antibodies to CarVY were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), GmbH, Germany (Catalogue Nos. AS-0148 and AS-0759, respectively). Leaf samples were extracted (1 g leaf/20 mL buffer) in phosphate buffered saline (0.14 M sodium chloride; 0.0015 M monobasic potassium phosphate; 0.0067 M dibasic sodium phosphate; 0.0027 M potassium chloride; 0.003 M sodium azide), pH 4, containing 5 % of Tween 20 and 2 % of polyvinyl pyrrolidone. The extracts were tested using a double antibody sandwiched enzyme-linked immunosorbent assay (DAS-ELISA) as described by Clarke and Adams (1977). To determine the efficacy of CarVY inoculation, non-transgenic inoculated and uninoculated controls were assayed in duplicate wells of a microtitre plate, using the CarVY specific DAS-ELISA, at 14 days post inoculation (dpi). Following determination of successful virus inoculation, each test plant including transgenic virus positive and negative controls (uninoculated) and non-transgenic positive controls and negative controls (uninoculated), were assayed in duplicate

wells of a microtitre plate. A buffer control was also included for normalisation of data. Substrate buffer (0.92 M diethanolamine; 0.003 M sodium azide; pH 9.8) was added to each well and incubated at room temperature for 1 hr. Absorbance values (A_{405nm}) were measured in a Multiscan plate reader (Labsystems, Finland) and samples with optical densities of more than twice those of the corrected healthy control optical densities were considered to be infected with CarVY. For comparison of inoculated and uninoculated carrot plant lines, five biological replications were assessed for each of the 6 transgenic lines and the wild type control. The General Linear Model (GLM) function and Tukeys comparison test, within MINITAB for Windows Version 14 (Minitab® Inc, 2003), was used to determine the occurrence of significant differences between optical density means ($P<0.05$) for each line tested. Different letters were assigned to OD readings to indicate those lines that differed significantly from each other following comparison of significant differences.

6.3 Results

6.3.1 Plant transformation and regeneration

Transformation of carrot tissue, with the AGL1 strain of *A. tumefaciens* harbouring the ihpRNA construct pPOPOV-CarVY, resulted in a transformation efficiency rate of 0.85 % for the carrot cultivar Crusader, and the generation of six independent transgenic carrot plants (Table 6.1). The transformation of Stefano petioles with AGL1 harbouring pPOPOV-CarVY resulted in the production of 2 kanamycin resistant callus explants, but subsequent regeneration into mature plants from this callus tissue was not achieved (Table 6.1). The transformation of the AGL1 strain of *A. tumefaciens* harbouring the pPOPOV-CeMV to petioles of celery cultivars Summit and Tendercrisp was unsuccessful, with 0 % of transformed petioles generating kanamycin resistant callus (Table 6.1).

Table 6.1 Transformation efficiency rates of carrot and celery tissue transformed with *A. tumefaciens* strain AGL1 harbouring pPOPOV-CarVY and pPOPOV-CeMV.

Engineered *A. tumefaciens* strain AGL1	Species	Cultivar	No. petioles used	No. kanamycin resistant calli generated	No. transgenic lines generated
pPOPOV-CarVY	Carrot	Crusader	700	8 (~1%)*	6 (0.85%)
		Stefano	1000	2 (0.2%)	0 (0.0%)
pPOPOV-CeMV	Celery	Summit	1150	0 (0.0%)	0 (0.0%)
		T'crisp	1150	0 (0.0%)	0 (0.0%)

Numbers in brackets = no. transgenic calli or plants/no. petioles (as a percentage).

6.3.2 Acclimatisation of tissue culture plants in the glasshouse

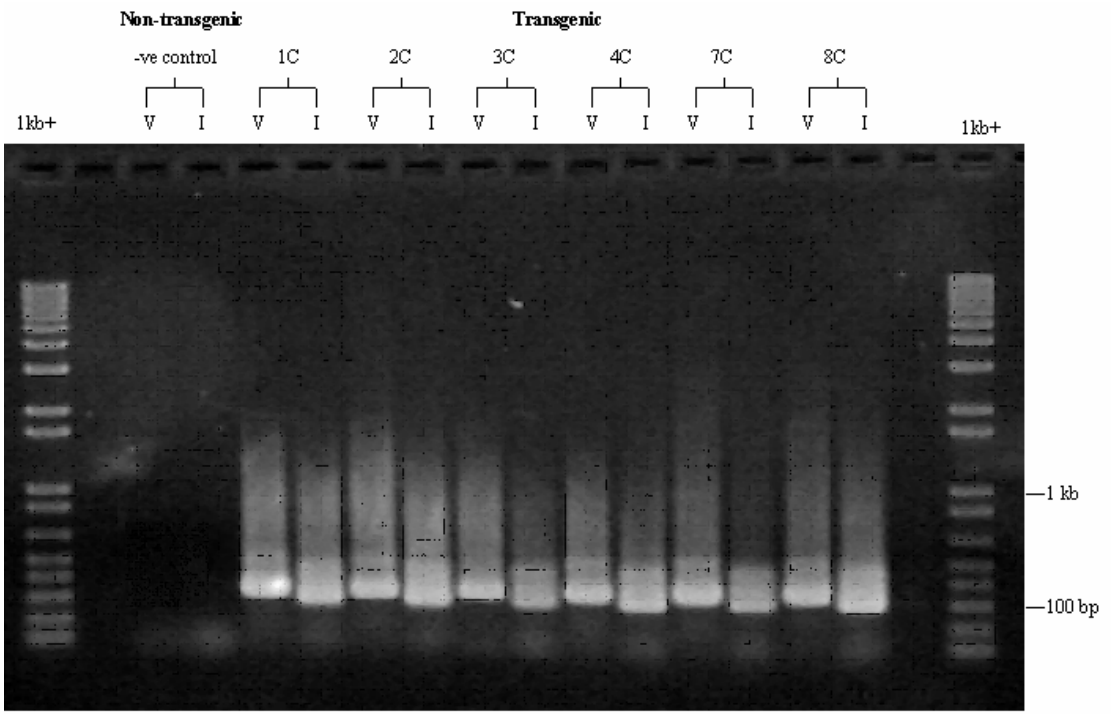
Following clonal multiplication of the six transgenic carrot lines of cultivar Crusader (Lines 1-6), transformed with the pPOPOV-CarVY resistance construct, 10 plants of each independent line were successfully acclimatised to the glasshouse (Figure 7.1). As controls, 10 non-transgenic Crusader plants were similarly acclimatised and compared to the transgenic carrot lines following subsequent inoculation with buffer (5 plants) and CarVY (5 plants).

Figure 6.1. Acclimatisation of carrot plants of cultivar Crusader to glasshouse conditions. I. One day following deflasking from tissue culture container; the cup provides a high humidity environment. II. Four weeks following deflasking; growth of new petioles and leaves are clearly evident. III. Population of six transgenic lines (10 plants per line) and non-transgenic control plants (10 plants).

6.3.3 Analysis of transgenic plants

6.3.3.1 Analysis by PCR

Examination of the transgenic carrot lines by PCR amplification, using primers pairs targeting both the CarVY insert (pPOPOVCarVYF – pPOPOVCarVYR) and the intron spacer region (pPOPOVCarVYintronF – pPOPOVCarVYintronR), indicated that all six transgenic lines contained one or more copies of the pPOPOV-CarVY ihpRNA construct (Figure 6.2).



V = PCR fragment generated using antisense virus insert targeting primer pair (pPOPOVF – pPOPOVR); I = PCR fragment generated using pKannibal intron targeting pair (pPOPOVCarVYintronF – pPOPOVCarVYintronR).

Figure 6.2. PCR analysis of six transgenic carrot lines harbouring pPOPOV-CarVY ihpRNA construct.

6.3.3.2 Immunological assays

At 14 days following the inoculation of CarVY sap onto the 6 transgenic lines, DAS-ELISA assessment with CarVY-specific antisera indicated that non-transgenic positive and negative controls did not contain sufficient virus for detection. Further assessment at 21 dpi and 28 dpi also indicated virus was either not present in plant tissue or at levels below detection by DAS-ELISA. Subsequently, plant inoculation with CarVY sap extract was carried out weekly intervals for three weeks to increase inoculum pressure on both the transgenic and non-transgenic plants. DAS-ELISA assessment of non-transgenic inoculated and uninoculated plants at 49 dpi showed some degree of resistance following inoculation with CarVY for all six transgenic lines (Figure 7.3). For all plant lines, including the non-transgenic control, inoculated plants produced a higher OD reading than the uninoculated plants. However, OD readings of inoculated transgenic plant lines were significantly lower than readings for inoculated non-transgenic controls. Transgenic plant line 3 had the lowest accumulation of virus as determined by DAS-ELISA and transgenic plant line 4 showed the least variation between uninoculated and inoculated plants.

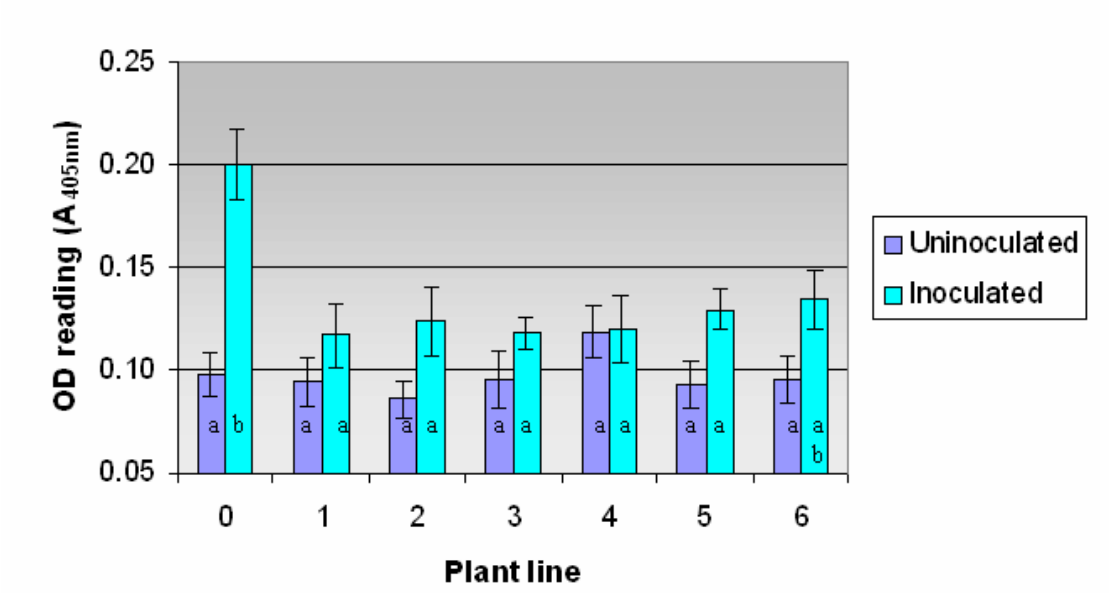


Figure 6.3. DAS-ELISA assessment of six transgenic carrot lines of cultivar Crusader harbouring pPOPOV-CarVY (0 = non-transgenic control).

6.4 Discussion

A resistant phenotype has been defined as one that had reduced levels of virus, versus an immunity or immunity-like phenotype where no virus was detected (Berger and German 2001). In this study we refer simply to the presence of resistance of a transgenic carrot line based on a negative or reduced optical density using ELISA when compared to healthy carrot control. More intensive work is required to demonstrate resistance or immunity and includes field assays and therefore is not considered a requirement of this research project.

There are a number of encouraging results obtained from this research. We have generated six transgenic carrot plants of cultivar Crusader that contain transgenes encoding an ihpRNA containing CarVY sequence from the NIa cistron. All of these independently transformed plants show a degree of resistance to CarVY. We have shown that only a small region of a viral genome (approximately 300 nucleotides contained in pPOPOV-CarVY) is required to confer resistance to the transgenic plant. Using only small regions of the viral genome is environmentally desirable as it minimises the risk of viral recombination between the transgene and natural populations of plant viruses that may infect the transgenic potatoes in the field.

Over 2200 celery petiole pieces from Summit and Tendercrisp were exposed to the AGL1 strain of *A. tumefaciens* harbouring the pPOPOV-CeMV resistance construct and not one transformed celery plant was recovered. These findings are consistent with the findings in earlier chapters and it is evident that celery tissue has a toxic reaction to either the bacterium *A. tumefaciens* or some of the necessary reagents (ie. acetosyringone) which results in the extremely low transformation efficiency rates. The pPOPOV-CeMV was placed in to carrot tissue and is ready to be challenged for resistance.

Further work is required to better understand the nature and degree of CarVY resistance identified in this study. We would like to

1. determine the number of insertions of pPOPOV-CarVY ihpRNA constructs into the independent transgenic carrot lines by Southern blot analysis
2. use Northern blot analysis to detect siRNAs in the independent transgenic carrot lines that are produced as a result of PTGS,
3. and challenge the carrot lines that have been transformed with the pPOPOV-CeMV construct.

We have demonstrated the effectiveness of the CarVY resistance construct pPOPOV-CarVY to confer disease resistance in carrots. It is highly probable that the CeMV resistance construct pPOPOV-CeMV will confer similar resistance to CeMV in both carrot and celery. This technology can be applied to any carrot and celery cultivar on request from industry as the systems optimised in this study can be broadly applied .

6.5 References

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7.0 Technology transfer

Nina McCormick (PhD student) attended a conference in Spain and visited labs in France and England. Improvements and modifications identified from these visits were incorporated into the methodologies described in this report.

The industry liaison group has been kept informed on the progress of this project.

Results of this research have been presented in conferences, laboratory visits and published press articles. (See 6.2)

8.0 Publications/conferences

8.1 Conferences - Posters:

1. **McCormick, N.L.**, Ford, R., Taylor, P. and Rodoni, B. (2004) The development of virus resistant carrot genotypes using RNAi technology. *4th International Crop Science Congress,* Brisbane, Australia.
   Ref: www.cropscience.org.au/icsc2004/poster/3/8/679_mccormick.htm

2. **McCormick, N.L.**, Ford, R., Taylor, P., Isenegger, D., Rodoni, B. and Hutchinson, J.F. (2003) The development of virus resistant carrot and celery genotypes using gene silencing technology. *COMBIO*, Melbourne, Australia.

3. **McCormick, N.**, Ford, R., Taylor, P., Isenegger, D. and Hutchinson, J.F. (2003) The development of virus resistant carrot and celery genotypes using gene silencing technology. *7th International congress of Plant Molecular Biology.* ISPMB, Barcelona, Spain.

8.2 Conference Presentations (oral)

1. **McCormick, N.L.**, Ford, R., Taylor, P. and Rodoni, B. (2004) The development of virus resistant carrot genotypes using RNAi. *Proceedings of the School of Agriculture and Food Systems Postgraduate Conference*, The University of Melbourne, Australia.

2. **McCormick, N.L.**, Ford, R., Taylor, P., Isenegger, D., Rodoni, B. and Hutchinson, J.F. (2003) The development of virus resistant carrot and celery genotypes using RNAi. *Proceedings of the Tenth Annual Scientific Symposium of the Joint Centre for Crop Improvement,* Rutherglen.

3. **McCormick, N.L.**, Ford, R., Taylor, P. and Rodoni, B. (2003) Developing virus resistant carrot and celery genotypes using gene silencing technology. *Proceedings of the School of Agriculture and Food Systems Postgraduate Conference*, The University of Melbourne, Australia.

4 **McCormick, N.**, Ford, R., Taylor, P., Isenegger, D. and Hutchinson, J.F. (2003) The development of virus resistant carrot and celery genotypes using gene

silencing technology. *Seminar Series*, The University of Melbourne, Australia. Ref: http://events.unimelb.edu.au/eventid_24.html

8.3 PhD Thesis

Nina McCormick – Production and assessment of virus resistant carrot and celery cultivars for the fresh market. (In Prep).

8.4 Laboratory visits

A number of grower and industry groups have visited IHD and CSIRO including:

- The University of Melbourne (Institute of Land and Food Resources).
- IHD Open Days (2003).
- Gene Silencing: A new tool for developing virus resistant horticultural crops. NRE Hort Conference 2002, IHD, Knoxfield.

Staff associated with the project have also visited a number of reseaerch groups and laboratories during the course of this project:

- CSIRO Plant Industries, Canberra, Australia. Brendan Rodoni and Nina McCormick visited Peter Waterhouse who is a project collaborator and discussed the design of the virus resistance constructs and the glasshouse screening trials for virus resistance.
- Nina McCormick attended the 7th International congress of Plant Molecular Biology in Barcelona, Spain (June 2003).
- Sainsbury University, Norwich, United Kingdom. Nina McCormick visited David Baulcombe's lab and discussed new and emerging RNAi strategies that can be applied to vegetable crops including carrots and celery (July 2003)
- Cambridge University, London, United Kingdom. Nina McCormick visited Prof Jim Haseloff and discussed transformation and regeneration systems for plants and acquired some improved transformation constructs containing the GFP reporter gene.
- An Industry Liaison Committee meeting was held at the property of Tom Schruerers at Clyde, Victoria, to discuss the outcomes of the project and seek guidance from industry on how to preserve the transgenic plants and resistance constructs produced during the course of this project.

9.0 Recommendations

1. A complex system for the generation of transgenic carrot has been developed and is outlined in section 6.2. It is recommended that this methodology be used as a technology platform in future research projects involving genetic manipulation to introduce other attributes into carrots. Gene technology is the ideal way to conduct targeted breeding experiments as demonstrated with our research on virus resistance.

2. A complex system for the regeneration of transgenic celery has been researched and significant progress made in the optimisation of these procedures. It is recommended that further research should:

- Trial other strains of *A. tumefaciens* that may be more compatible with celery tissue
- Trial other types of resistance genes that can be used as selectable markers, such as herbicide resistance genes. The mode of action of these selectable markers may prove to be less toxic to the celery tissue
- Consider alternative methods of introducing transgenes into celery tissue. Although there are some distinct advantages in *A. tumefaciens* mediated transformation (single gene copy number) other methods of transformation (ie particle bombardment) should be tried as these technologies may be successful at introducing transgenes into celery.

3. The carrot lines transformed with the pPOPOV-CarVY transgene do confer resistance to CarVY in glasshouse trials. It is recommended that a) seed is harvested from the 6 transgenic lines of carrot cultivar Crusader, and b) the virus resistance plasmids (pPOPOV-CarVY and pPOPOV-CeMV) are stored indefinitely as these constructs can be used on other carrot and celery cultivars and breeding lines.

4. It is strongly recommended that the CarVY resistant carrot lines transformed with pPOPOV-CarVY be screened in field trials to determine agronomic performance and assess the performance of the conferred virus resistance to field challenge of virus inoculum. Any field assessment of engineered lines needs to be done with the approval of the Office of Gene Technology Regulator.

5. The recommendations proposed will benefit industry in different ways, depending on the intended use of the carrot and celery germplasm;

- *Existing cultivars.* The advantage to industry of engineering existing cultivars is that growers are already familiar with agronomic attributes of the cultivar and therefore a minimum amount of additional agronomic research is required.
- *Advanced breeders lines.* Engineered lines can be assessed in precisely the same manner as any conventionally bred cultivar is.
- *Parental lines.* Conventional breeders could primarily use engineered lines. In previous research we have demonstrated that newly introduced genes are inherited and developed methods to select transgenic seedlings.

6. The industry needs to be aware of the advantages and disadvantages of gene technology so that meaningful decisions can be made about future investments. The two websites listed below are suggested as general reference points; AgBioView (http://www.agbioworld.org) and AgNet (http://www.plant.uoguelph.ca/safefood).