TISSUE CULTURE AND <u>AGROBACTERIUM</u>-MEDIATED TRANSFORMATION OF CARROT (<u>DAUCUS CAROTA</u> L.)

by

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Tissue Culture and Agrobacterium - Mediated

Transformation of Carrot (Daucus carota L.)

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Abstract

Transgenic carrot (*Daucus carota* L.) plants were obtained using disarmed *Agrobacterium tumefaciens* strains EHA 105 (leucinopine) and MOG 101 (nopaline). Each of the two strains harboured a binary plasmid containing either an acidic chitinase gene from petunia (pMOG196), or a basic chitinase gene from either tobacco (pMOG198) or bean (pGA492-CHN) driven by the constitutive 35-S promoter from Cauliflower Mosaic Virus (CaMV). In addition, the neomycin phosphotransferase (NPT II) gene from *Tn*5 encoding kanamycin resistance was present. The influence of the *Agrobacterium* strain, plasmid, carrot cultivar, age of explant, and co-cultivation time were evaluated. The highest frequency of transformation (12.1%) based on production of somatic embryos on Murashige and Skoog medium (MS) with 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 mg L^{-1} of kanamycin, was obtained with epicotyl segments of the cultivar Nanco cocultivated for 2 or 3 days with the supervirulent *A. tumefaciens* strain EHA 105. Suspension cultures were also initiated from the embryogenic calli in liquid MS medium with 0.5 µM 2,4-D and 50 mg L^{-1} of kanamycin. Plantlet development occurred within 2-4 months after plating either calli or cell suspensions onto MS medium without growth regulators or kanamycin; excised shoots were rooted on MS medium containing kanamycin (50 mg L^{-1}) before transferring to soil. Transformation was confirmed by PCR amplification of the NPT II coding region and by Southern hybridization analysis using an 800 bp Digoxigenin-UTP labelled probe specific for the NPT II gene. A single hybridizing band was seen indicating the integration of one T-DNA copy in the genome of the transgenic plants.

Dedication

To my parents and my husband Chris for their understanding, patience, and
encouragement.

Acknowledgments

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Chapter I

Introduction

Carrot (Daucus carota)

Carrot (*Daucus carota* L. subsp. *sativa*) is a member of the family
Umbelliferae, which also includes celery, parsnips, and parsley. Carrots are
widely grown for fresh market use and for processing. Economically, carrots
rank among the top 10 of the most important vegetable crops, with an annual
production of 7.72 x 10^6 tons in North America and Europe (Peterson and Simon
1986; Wijbrandi and de Both 1993). Carrots are an excellent source of vitamin A
and also contain considerable amounts of vitamin B_1 and C and an essential oil,
rich in vitamin E. Carrots also have large amounts of carbohydrates and are low
in protein and lipids (Ammirato 1986). They are also grown for their seed, since
extracts provide an oil that is used in flavouring and in the production of
perfumes (Ammirato 1986).

All cultivated carrots are forms of the wild Queen Anne's Lace (*D. carota*
subsp. *carota*). The species is diploid, with nine pairs of chromosomes (n = 9,
2n = 18) (Simon 1984; Sung and Dudits 1981). Carrot is typically biennial,
although annual forms are known. The plant produces a rosette of leaves and a
fleshy taproot during the first growing season, and the flower stalk and seed are
produced in the second year. The taproots need a cold period, or vernalization,
in order to sprout and flower in the second season. The carrot umbel is a
compound inflorescence, containing flowers arranged in umbellets. The flowers
are mainly perfect (male flowers occur frequently in high order umbels), are
small, and bear white petals. The seed is a mericarp, one-half of a dry,

indehiscent fruit. Both self- and cross-fertilization occur simultaneously in an umbel. Cross-fertilization is achieved by house flies.

Carrot varieties are typically classified according to the colour of the root (red, orange, or white), its shape (blunt, or pointed), and its length (short, medium, or long). Cultivars with greater uniformity of colour and shape are used for the fresh market trade, whereas uniform colour in the roots and high yield of large roots are required for processing.

Over the years, approaches to carrot breeding have drastically changed. Early methods included mass selection or combined mass pedigree selection, while recent methods involve inbred lines and hybridizations to produce F1 hybrids, based on male-sterile lines (Ammirato 1986; St. Pierre and Bayer 1991). The objectives of carrot breeding are to increase yield, improve root shape and colour, increase earliness, top-root ratio, and resistance to bolting, and improve quality characteristics and disease resistance.

Tissue culture of carrot

Carrot is considered to be one of the best model organisms from the standpoint of regenerative potential in tissue culture (Sung and Dudits 1981). In general, two regeneration pathways exist for plants, one being organogenesis and the other somatic embryogenesis. Regeneration via formation of adventitious shoots, induced either on callus tissue or directly on a cultured explant such as a leaf, hypocotyl or cotyledon is organogenesis. In somatic embryogenesis, undifferentiated cells develop into embryo-like structures through a sequence of events not unlike the development of zygotic embryos. These somatic embryos can germinate and grow into a mature plant (Sung and Dudits 1981; Wijbrandi and de Both 1993). Regeneration in carrot proceeds primarily through embryogenesis, in which the developmental sequence starts

from callus and progresses sequentially through globular, heart and torpedo-shaped embryos and into the cotyledonary stage (Ammirato 1986; Sung and Dudits 1981). Somatic embryos can be grown individually and freely floating in a small volume of liquid medium, and from them, large numbers of plants can be grown (Ammirato 1986).

Tissue culture thus offers two main advantages in a breeding program: regeneration can yield plant materials which are superior in uniformity, and these plants can be produced in higher numbers than from natural plant processes.

Somatic embryogenesis

a) Callus and regeneration

Somatic embryos of carrot can be produced by a relatively simple manipulation of culturing conditions. This generally involves placement of an explant, from aseptically germinated individual seeds, on a solid or semi-solid culture medium with the required nutrients and growth regulators. The most commonly used media are Murashige and Skoog (MS) medium supplemented with 0.5, 2.3, or 4.5 μM 2,4-D; MS medium with 4.5 μM 2,4-D and 0.1 μM kinetin or 0.44 μM benzyladenine; and Uchimya and Murashige (UM) (Scott and Draper 1987) and B5 medium (Thomas *et al.* 1989) with 9.0 μM 2,4-D. Some of the cells in the explant tissue respond to the new environmental conditions and develop into a rapidly proliferating, undifferentiated mass of cells (callus) (LoSchiavo *et al.* 1991; Zimmerman 1993). The callus requires subculturing at about 4-week intervals (Smith 1988). The callus tissue can also be removed from the solid medium and grown in liquid medium to produce suspension cultures. The cells grow faster in a liquid cell suspension and thus require more frequent subculturing (at 1- to 3-week intervals) (Smith 1988).

Within the dividing callus, some cells acquire totipotency, and in the presence of an auxin, such as 2,4-D, give rise to special clusters of cells termed 'proembryogenic masses' (PEM) which represent the first embryonal stage (LoSchiavo *et al.* 1991; Zimmerman 1993). These cell clusters can be selected out of the total population by sieving the culture and/or by low-density subculture into fresh medium (Ammirato 1986; Liyanage and Kurata 1992; Sung and Dudits 1981). The resulting enriched population is over 90% embryogenic and relatively synchronous in development (Zimmerman 1993). In the auxin-containing medium, proembryos can only advance up to the globular stage. Subculture of this population produces many other proembryos, thus providing fresh material for the regenerative process to continue (Ammirato 1987).

It is believed that in the continued presence of auxin, the PEMs within the culture synthesize all of the gene products that are necessary to complete the globular stage of embryogenesis (LoSchiavo *et al.* 1991). The PEMs also contain many other gene products whose continued presence generally inhibit the progression of the embryogenesis cycle (Zimmerman 1993). In order for the PEM to grow and mature, the auxin has to be depleted from the medium so that inactivation of a number of genes occurs and the embryogenesis program can proceed (LoSchiavo *et al.* 1991; Zimmerman 1993).

Globular embryos are first detected about 6 days following low-density subculture into auxin-free medium (Sung and Dudits 1981; Zimmerman 1993). After 2 to 3 additional days of isodiametric growth, the globular stage is followed by an oblong stage (Schiavone and Cooke, 1985), the globular-to-heart transition. This phase is clearly marked by the outgrowth of the two cotyledons, the elongation of the hypocotyl, and the beginning of radicle development (Zimmerman 1993). The embryo develops through the torpedo stage, and approximately 3 weeks after induction, plantlets can be identified that contain

green cotyledons, elongated hypocotyls, and developed radicles. These plantlets can be transplanted to solid medium or soil for development into whole plants.

b) Protoplasts

Protoplasts are produced using cellulases and pectinases which hydrolyze the cell wall from cells within a plant organ or in suspension cultures (Sung and Dudits 1981). Carrot protoplasts have been isolated from a variety of sources, including root tissue, callus tissue, suspension cultures, and from PEMs in suspension cultures (Ammirato 1986). The most efficient yields result from the use of actively dividing embryogenic suspension cultures and from maturing carrot somatic embryos (Ammirato 1986). The protoplasts regenerate a cell wall upon plating on appropriate tissue culture medium. For callus development, the tissue culture medium is supplemented with 2.3 µM 2,4-D, and plants can be regenerated in the same manner as from tissue explants (Ammirato 1986). Carrot protoplasts can also be directly grown into somatic embryos by adding 1µM napthaleneacetic acid and 0.5 µM zeatin to the culture medium, therefore, regeneration can occur without advancing from a callus phase (Ammirato 1986). In general, plant regeneration efficiency from protoplasts is low in comparison to regeneration frequency from explant tissues.

Somaclonal variation

'Somaclonal variation' is the term used to describe genetic changes which arise during tissue culture, such as alterations in chromosome number and karyotype and single gene changes (Scowcroft *et al.* 1987). Somaclonal variation can potentially generate substantial genetic variability in plants regenerated from tissue culture. It has been reported that carrot cells exhibit

substantial variation that is heritable, which can provide useful variant carrot cells and plants (Ammirato 1986; Sung and Dudits 1981).

A wide range of carrot variants have been produced through the use of a number of selection procedures. These include cell lines resistant to inhibitors, such as amino acid analogs (e.g., 5-methyltryptophan and S(2-aminoethyl)-L-cysteine), purine and pyrimidine analogs (e.g., 5-fluorouracil) and antibiotics (e.g., cycloheximide) (Ammirato 1986; Sung and Dudits 1981). Colour variants have also been isolated which produce purple or albino plantlets (Sung and Dudits 1981). These variant carrot cells hold much promise for the production of secondary products. Also of interest are variant lines that may lead to carrot varieties with increased tolerance to environmental extremes, such as temperature, drought or salinity.

An overview of genetic engineering

Over the past decade, advances in genetic engineering at the cellular level have made significant strides to genetically modify and improve agricultural plants. Recombinant DNA techniques for the introduction and expression of one or several genes, derived from similar or non-related organisms, are now available. In some cases, these genes encode agronomically useful characters.

The introduction and expression of foreign DNA into stably transformed plants is now routine for several model species, such as tobacco and *Arabidopsis*, and for major crop species such as cucumber, potato, pea, oilseed rape and sugar beet (D'Halluin *et al.* 1992; Komari 1989; Puonti-Kaerlas *et al.* 1989; Radke *et al.* 1988; Sarmento *et al.* 1992; Sheikholeslam and Weeks 1987; Tavazza *et al.* 1988).

The production of transgenic plants requires several critical components: (1) introduction and stable integration of the DNA into the plant chromosomes,

(2) selection and regeneration of a reproductively competent transgenic plant, and (3) analysis and verification of gene expression of introduced genes in the transformed plant and its progeny. This molecular breeding has already provided transgenic plants which are resistant to herbicides (Drögue *et al.* 1992; Gasser and Fraley 1989), insects (Fraley 1992; Gasser and Fraley 1989; Goldburg and Tjaden 1990), bacteria (Düring *et al.* 1993; Harms 1992) and viruses (Beachy *et al.* 1993; Fischoff 1988; Harms 1992). Such an approach could also be used to enhance plant protection against fungal diseases.

Agrobacterium tumefaciens

The most frequently used method for DNA transfer into plants is through the soil bacterium *Agrobacterium tumefaciens* (Cramer and Radin 1990; Gasser and Fraley 1989). *A. tumefaciens* causes crown gall disease on a wide range of dicotyledonous plants (Agrios 1988; Hooykaas and Schilperoort 1992; Kerr 1987). This bacterium has evolved a complex mechanism for transfer and integration of a defined segment of DNA (T-DNA) from its tumor-inducing (Ti) plasmid into the nuclear genome of plant cells (Horsch *et al.* 1987; Hooykaas and Schilperoort 1992; Kerr 1987; Potrykus 1990; Weissinger *et al.* 1988). The transfer of the T-DNA is mediated by genes in another region of the Ti-plasmid, referred to as the *vir* region. These virulence genes are not transferred with the T-DNA but act in *trans* to cause the transfer (Horsch *et al.* 1988; Yoneyama *et al.* 1993; Weissinger *et al.* 1988). The T-DNA is defined by a 25-base pair sequence, the border sequence, which is the recognition site for specific endonucleases which are involved in excision of the T-DNA (Cramer and Radin 1990; Horsch *et al.* 1988).

Wounded plant cells produce phenolic compounds, such as acetosyringone, hydroxyacetosyringone, guiacol, coniferyl alcohol and sinapinic

acid, which play a role in the bacterium-host cell recognition mechanism (Binns and Thomashow 1988; Cramer and Radin 1990; Hooykaas and Schilperoort 1992; Spencer and Towers 1988; Yoneyama and Anzai 1993). Such compounds are believed to be involved in the induction of the *vir* region on Ti-plasmids, leading to the production of endonucleases which nick the bottom strands of the T-DNA border sequences. This generates free, linear, single-stranded copies (T-strand) of the T-DNA, which are transferred into plants from the bacterial cells (Binns and Thomashow 1988; Hooykaas and Schilperoort 1992; McLean *et al.* 1993). Finally, the T-strands are incorporated covalently into the plant chromosomes at random sites as a double-stranded DNA insert. It has been noted that the plant sequences adjacent to the T-DNA inserts are often A-T rich (Binns and Thomashow 1988).

Because the excision event of the T-DNA is a key step in the transfer of DNA from *A. tumefaciens* into the plant genome, the addition of exogenous acetosyringone may stimulate the bacterium to transfer the T-DNA more efficiently. This approach has been useful in plant systems that have low rates of transformation, such as *Arabidopsis* (Sheikholeslam and Weeks 1987), *Antirrhinum majus* and *Glycine max* (Godwin *et al.* 1991), and *Rubus* species (Hassan *et al.* 1993). It has been reported that the addition of acetosyringone is useful only if the plant does not produce one of the many phenolic compounds that can be recognized by the bacterium, or if the co-cultivation period is too short to allow induction, chemotaxis, attachment and gene transfer (van Wordragen and Dons 1992). In some cases, acetosyringone has been found to be disadvantageous, such as in *Pisum sativum* (De Kathen and Jacobsen 1990). In other cases, acetosyringone reduces the genotype effect (van Wordragen and Dons 1992), and sometimes there can be no effect (Godwin *et al.* 1991; Pawlicki *et al.* 1992; van Wordragen and Dons 1992).

The T-DNA contains several genes, including those for the production of phytohormones (an auxin and a cytokinin) which induce the formation of the characteristic tumours. In addition, genes coding for the production of opines, which are amino acid analogues that the bacteria subsequently utilize as a source of nutrition, are also present (Hooykaas and Schilperoort 1992; Yoneyama and Anzai 1993; Wijbrandi and de Both 1993). The Ti-plasmids of *Agrobacterium* have been divided into four groups based on the opines produced in tumours: octopine, nopaline, leucinopine and D,L-succinamopine (Melchers and Hooykaas 1987; Horsch *et al.* 1988). Generally, virulence and host range of the bacterium vary depending on the opines produced. Octopine strains have often been found to be less virulent and to have a narrower host range than nopaline or succinamopine strains (Godwin *et al.* 1991; Komari 1989). Several reports indicate that the leucinopine and the succinamopine strains are supervirulent, since they provide the highest transformation in all plant species tested (Komari 1989; Puonti-Kaerlas *et al.* 1989; van Wordragen and Dons 1992).

The phytohormone biosynthetic genes can be removed to delete the oncogenic phenotype, while still leaving the ability of *Agrobacterium* to transfer DNA into plant chromosomes intact (Fischhoff 1989; Yoneyama and Anzai 1993; Wijbrandi and de Both 1993). In these disarmed strains, "foreign" genes, which usually include a selectable marker, and a foreign protein, can be introduced. *A. tumefaciens* transformation is mostly used with explants ('leaf disc method', Klee *et al.* 1987) and protoplasts (Bower and Birch 1993).

Once transferred to the plant, one to several copies of the T-DNA are covalently inserted at single or multiple sites in the host chromosome (Binns and Thomashow 1988; Cramer and Radin 1990; Hooykaas and Schilperoort 1992). The foreign DNA, once integrated, assumes characteristics of typical eukaryotic

chromatin and is stably maintained and transmitted to progeny as typical Mendelian traits (Cramer and Radin 1990; Rogers *et al.* 1988).

Vector systems

The structure of both the T-DNA and Ti-plasmids have been extensively modified to enhance their usefulness as plant transformation vehicles. Genetic experiments have established that none of the genes or DNA sequences within the T-DNA are necessary for the transfer process. The only requirement in *cis* for T-DNA delivery is the 25-bp border repeats, and only the right border is absolutely required since it acts as a recognition signal for the transfer apparatus (Cramer and Radin 1990; Hooykaas and Schilperoort 1992; Horsch *et al.* 1988). Based on these findings, vector systems for the transformation of plants have been developed. These vectors have been designed so that replication can occur in *Escherichia coli*, where recombinant manipulations are easily handled, and can then be transferred into *A. tumefaciens* in preparation for transfer into plants. The vectors fall into two categories: (1) *cis* systems (co-integrating vectors) in which new genes are integrated within the T-DNA of a resident Ti-plasmid via a single homologous recombination event (Herrera-Estrella and Simpson 1988; Hooykaas and Schilperoort 1992), (2) *trans* systems (binary vectors) in which new genes are cloned into an autonomously replicating plasmid distinct from that carrying the *vir* genes necessary for transformation (Cramer and Radin 1990; Hooykaas and Schilperoort 1992; Horsch *et al.* 1988).

Leaf disc transformation

Two basic approaches have been used to transform plant tissues using *Agrobacterium*: co-cultivation of regenerating protoplasts and the leaf disc procedure (Klee *et al.* 1987; Fraley *et al.* 1985). The former has some

limitations, since it is only applicable to plant species with well-developed protoplast regeneration systems. In addition, because the co-cultivation procedure is extended it is possible to have abnormalities induced through prolonged cell culture (Fraley *et al.* 1985). In the leaf disc transformation method, gene transfer, selection, and regeneration of leaf explants are sychronized in an efficient process (Rogers *et al.* 1988; Horsch *et al.* 1988). Infection of sterile leaf discs by brief incubation (1-60 min depending on the species) in a suspension of *A. tumefaciens* is conducted. The discs are then allowed to co-cultivate with the *Agrobacterium* for a period of time (2-10 days depending on the species) and then washed to eliminate most of the bacteria. The co-cultivated explants are placed on regeneration media containing an antibiotic or herbicide to allow growth of transformed tissues and antibiotics to prevent growth of the bacteria (cefotoxamine, carbenicillin). Within a few weeks, depending upon the plant species, small shoots develop at the edges of the discs, which can be excised and transferred to the appropriate medium to allow growth of plantlets (Herrera-Estrella and Simpson 1988; Rogers *et al.* 1988; Horsch *et al.* 1988). This procedure is especially suited for *Arabidopsis* (Sheikholeslam and Weeks 1987), carrot (Pawlicki *et al.* 1992), cucumber (Chee 1990; Sarmento *et al.* 1992), lettuce (Torres *et al.* 1993), muskmelon (Fang and Grumet 1990), pea (De Kathen and Jacobsen 1990; Puonti-Kaerlas *et al.* 1989), tobacco (Horsch *et al.* 1985), and tomato (Fillatti *et al.* 1987; Horsch *et al.* 1985).

The leaf-disc technique can be easily used with other explants, such as stem sections, cotyledon sections etc., depending upon the explant which is most suited for regeneration of plants (Herrera-Estrella and Simpson 1988).

Selectable marker genes

Transformed plant cells following *Agrobacterium* infection were initially identified by tumorous gall formation or by hormone-independent growth in culture (Klee *et al.* 1987). Disarming of the T-DNA required the development of alternative selectable markers that would be expressed in plants to aid in the identification of transformed cells or tissues. Several dominant selectable markers have proved useful in transformation studies.

a) Antibiotic resistance

The most widely used and effective antibiotic marker is the *Tn5*-derived neomycin phosphotransferase, type II (NPT II) enzyme. This enzyme detoxifies aminoglycoside compounds such as kanamycin and G418 by phosphorylation (Cramer and Radin 1990; Klee *et al.* 1987). Kanamycin is inhibitory to plant cell growth, and NPT II expression provides a high level of resistance to transformed cells, plants, and seeds (Cramer and Radin 1990). In the presence of the antibiotic, the untransformed cells die while the transformed cells grow and multiply. Expression in plants has been possible through the construction of chimeric genes in which the structural sequences of the gene encoding NPT II have been fused to 5' and 3' regulatory sequences, such as the constitutive NOS (nopaline synthase) promoter and terminator from *Agrobacterium*, which enable expression in plant tissues (Fisk and Dandekar 1993). In addition, sensitive assays for neomycin phosphotransferase activity are available for crude cell extracts (Reiss *et al.* 1984; McDonnell *et al.* 1987; Nagel *et al.* 1992).

Other antibiotic resistance genes have also been used successfully for plant transformation, and these include hygromycin resistance (Waldron *et al.*

1985), gentamycin resistance (Hayford *et al.* 1988), streptomycin resistance (Jones *et al.* 1987), and bleomycin resistance (Hille *et al.* 1986).

b) Herbicide tolerance

Herbicides represent a group of compounds that are potent metabolic inhibitors, since they affect a particular step in the biosynthetic pathway for various amino acids (Fisk and Dandekar 1993). They can be used as an alternative for screening for transformed cells. Engineering of herbicide tolerance has been achieved by two approaches. One approach involved altering the level (i.e. overexpression) and sensitivity (i.e. mutant genes) of the target enzyme for the herbicide. An example of this is the incorporation of the mutant *aro*A gene, which confers tolerance to glyphosate, into tomato (Fillatti *et al.* 1987). The second approach incorporates a gene for an enzyme that inactivates the herbicide (Fraley 1992; Gasser and Fraley 1989; Shah *et al.* 1986). Resistance to gluphosinate and bromoxynil has been achieved by the introduction of bacterial genes encoding enzymes that inactivate the herbicides by acetylation or nitryl hydrolysis, respectively (Gasser and Fraley 1989).

Genetic transformation of carrot

Genetic improvement of carrot and the development of new varieties has relied greatly on standard breeding techniques. However, this area has been dramatically broadened by the development of tissue-culture techniques and genetic engineering. These approaches should now permit the introduction of new traits into existing varieties, while still retaining much of the characteristic properties of the variety, and also avoid the genetic reassortments that occur during plant breeding. Two main methods have been used to transfer specific genes into carrot cells: (a) direct gene transfer and (b) indirect gene transfer.

The direct gene transfer method utilizes protoplasts, and the indirect gene transfer utilizes *Agrobacterium*.

Genetic transformation using carrot protoplasts

Due to the absence of a cell wall, protoplasts are amenable to various techniques of genetic manipulation, particularly those based on fusion of protoplasts from different sources. Such fusion allows for the combination of two complete genomes, partial genome transfer, or transfer of organelles. Protoplast fusion can be achieved by either chemical or electrical means. The mechanism of fusion is thought to occur by lateral diffusion of proteins in contacting membranes, thereby creating lipid-rich domains which are destabilized and fused (Lindsey and Jones 1989).

Carrot protoplasts have been successfully employed in fusion experiments and have resulted in intraspecific (*D. carota* resistant to 5-methyltryptophan), interspecific (*D. capillifolius* L.), and intergeneric (*Aegopodium podagraria* L. and *Petroselinum hortense* J. Hill) somatic hybrids. All of these fusions resulted in complementation of specific traits, such as resistance to amino acid analogues or restoration of albino mutants (Ammirato 1986; Sung and Dudits 1981).

Other methods have been developed by which direct DNA uptake occurs in protoplasts by chemical and electrical means. Exposure of protoplasts to chemicals, such as polyethyleneglycol (PEG) and polyvinyl alcohol (PVA), or to high voltage electric pulses, cause the plasma membrane to destabilize and allows entry of DNA into the cell (Bower and Birch 1993; Gasser and Fraley 1989). In carrot, these techniques have been utilized mainly to study gene regulation, such as the effects of promoters, enhancer elements, and introns, which influence transcriptional efficiencies. These studies have been possible

by coupling the sequences of interest with the coding regions of reporter genes such as the firefly luciferase (*lux*), neomycin phosphotransferase (NPT II), chloramphenicol acetyl transferase (*cat*) and B-glucuronidase (GUS) (Bower and Birch 1993; Rasmussen and Rasmussen 1993). Recently, a gene for herbicide resistance was introduced into carrot via direct gene transfer (Drögue *et al.* 1992). The resultant transgenic plants contained the phosphinothricin-*N*-acetyltransferase gene and were shown to be resistant to the herbicide L-phosphinothricin.

Agrobacterium-mediated transformation of carrot

Direct gene transfer into protoplasts generally is useful for transient gene expression studies. Transformation by *Agrobacterium* provides genetically stable gene introduction (Bower and Birch 1993). Both *Agrobacterium tumefaciens* (crown gall pathogen) and *A. rhizogenes* (hairy root pathogen) have been used as vehicles for the introduction of foreign genes into carrot. In 1985, Tepfer inoculated carrot roots with wild *A. rhizogenes* to produce transformed root tissue from which plants were successfully regenerated via somatic embryogenesis. Although these plants had altered root and leaf morphology, they were used to quantify rates of transfer of introduced genes through successive generations. *A. rhizogenes* was also used to introduce the maize transposable element *Ac* into carrot (Van Sluys and Tempe 1987). Upon regeneration of plants, the characteristic hairy root phenotype developed, but this did not impede study of *Ac* activity.

To date, four reports describe *A. tumefaciens*-mediated gene transfer into carrot. Scott and Draper (1987) utilized proembryogenic suspension cells and inoculated them with a non-oncogenic *A. tumefaciens* strain C58C1 carrying the plasmid pGV3850::1103. The plasmid contained a chimaeric kanamycin

resistance gene (nos-NPT II). Transformation efficiency was estimated to be 62-73%. Plants were regenerated from transformed cells via somatic embryogenesis in the presence of 100 mg L^{-1} kanamycin. Southern blot analysis showed that copy number ranged from one to eight. Western blot analysis confirmed the expression of the NPT II gene and indicated that NPT II was produced as a single, full-length polypeptide (Scott and Draper 1987).

A second report used callus cells and transformed them with several strains of *A. tumefaciens* which possessed the same C58 chromosomal background (Wurtele and Bulka 1989). Each strain contained binary vector pGA472 which comprised the nos-NPT II construct. Callus pieces (1-month-old) were divided into 0.5 g (fresh-weight) aliquots and mixed with a suspension of *A. tumefaciens*. Calli were co-cultivated for 3 days and were subsequently transferred to regeneration medium. Plants were regenerated via somatic embryogenesis on medium containing 300 mg L^{-1} kanamycin. The effects of *p*-hydroxybenzoic acid, acetosyringone, and mechanical wounding of the cells at the time of co-cultivation were studied. The results showed that these treatments had little or no effect on the timing or frequency of kanamycin-resistant clump formation (Wurtele and Bulka 1989). Southern blot analysis, using the binary vector as a probe, showed that the transgenic plants contained 1 to 15 copies of the introduced gene (Wurtele and Bulka 1989).

A third report used hypocotyl segments from sterile 1-week-old seedlings as the explant sources (Thomas *et al.* 1989). The explants were preincubated on tissue culture (B5 medium with 9µM 2,4-D) for 2 days prior to infection with *A. tumefaciens* LBA4404 containing CaMV 35S promoter-GUS construct on a binary vector (pRGUSII). The plasmid also contained the NPT II gene for kanamycin resistance. The results showed that preincubation of explants was essential, since no transformation occurred in the absence of the preculture

treatment (Thomas *et al.* 1989). Different carrot varieties were surveyed and it was found that production of kanamycin resistant calli was variety-dependent and ranged from 0.9 - 5.8%. The transformed calli (up to 1 cm in diameter) were used to establish suspension cultures, and transformed plants were regenerated via somatic embryogenesis. NPT II and Southern blot analyses confirmed that selected lines were transformed, with 1-3 copies of the GUS gene (Thomas *et al.* 1989).

Recently, Pawlicki *et al.* (1992) described the factors influencing *A. tumefaciens*-mediated transformation of carrot. The parameters studied were carrot variety, age and type of explants, co-cultivation and precultivation times, and effect of acetosyringone. Explants from five varieties were co-cultivated with a non-oncogenic *A. tumefaciens* strain C58C1 carrying the plasmids pGSTRN943 or pGSGluc1. Both plasmids contained a chimeric NPT II gene, and pGSGluc1 also contained the B-glucuronidase gene. Petioles, cotyledons, hypocotyls and roots were used as explant sources and were compared for their transformation efficiency. Co-cultivation periods were varied from 1 to 7 days, and precultivation periods (1, 2, 3, 7 days or 1 month) prior to infection, and the presence (100 µM) or absence of acetosyringone, were examined. Plants were regenerated from embryogenic calli (with 100 mg L^{-1} kanamycin) and rooted plantlets were potted and transferred to the greenhouse.

The results from this study showed that transformation efficiency was dependent on carrot variety, and ranged from 0-46.7%. Explant type was also found to be important, with petiole explants being more conducive to transformation than cotyledon, hypocotyl or root explants. The age of explants was also found to be a variable, with 3-4 week old seedlings being optimal. Co-cultivation periods of 2 or 3 days gave higher transformation frequencies than 1 or 7 days. Lastly, it was found that a preculture period or the presence of

acetosyringone did not influence transformation efficiency. Transformation was confirmed by histochemical detection of B-glucuronidase activity in transformed cells, and by Southern hybridization analysis.

Confirmation of transformation

Following transformation, the presence of the introduced gene requires confirmation. Two techniques can be used to confirm the presence of foreign genes in transgenic plant tissues: Southern Blot hybridization and Polymerase Chain Reaction (PCR). Expression of the introduced gene can be tested by Western blot analysis to detect the corresponding protein.

Southern blot hybridization

Southern analysis is used to determine the presence of specific sequences of DNA in gel-fractionated nucleic acid, and requires the following. First, the transfer of fractionated DNA to a support membrane by capillary action takes place, so that the relative position of the DNA remains unchanged. Second, the hybridization of the immobilized DNA to a labelled hybridization probe for the detection of specific DNA sequences is conducted (Sambrook *et al.* 1989; Scott 1988).

Genomic DNA (5-10 µg) is digested with one or more restriction endonucleases and is subsequently separated by agarose gel electrophoresis. The DNA in the gel is then denatured *in situ* by treatment with strong alkali, which renders the DNA single-stranded (Scott 1988). The DNA is then transferred to a solid support, usually a nitrocellulose filter or a nylon membrane. The relative position of the DNA fragments is preserved during the transfer to the membrane. Once transferred, the DNA is 'fixed' on the support by either baking of the filters (nitrocellulose) or by UV cross-linking (nylon). The immobilized

single-stranded DNA can then be hybridized with single-stranded, labelled probe DNA in solution. The probe binds only to DNA fragments having a high degree of homology. After hybridization, the filter is washed to remove any non-specifically bound probe and the labelled bands are located by exposure to X-ray film (Sambrook *et al.* 1989; Scott 1988).

Southern blot analysis may include endonuclease restriction with an enzyme which cuts twice within the plasmid to show the presence of the foreign gene, or with an enzyme that cuts once outside the gene of interest to yield restriction fragments corresponding to the site(s) of insertion. There are reports in which the first approach was used, where the DNA was digested with enzymes to excise the NPT II fragment to yield a band approximately 2.2 kb in size (Chee *et al.* 1989; Chee *et al.* 1990; Sarmento *et al.* 1992; Tavassa *et al.* 1988) or to excise the GUS gene (Hassan *et al.* 1993; Nehra *et al.* 1990; Torres *et al.* 1993). With this approach, copy number can be estimated by comparing hybridization signals in the transformed plants to those obtained from genomic reconstructions equivalent to a range of copy numbers (Sarmento *et al.* 1992). With the second approach, where the DNA outside the T-DNA region is digested with a single enzyme, a hybridizing fragment indicative of the chromosomal location of the inserted fragment within the plant genome should be detected. This method yields a single hybridizing band in plants containing a single copy of the gene, and multiple bands are seen for plants containing several copies. There are several reports in different plant species, including cucumber, muskmelon and tomato (Bernatzky and Tanksley 1986; Chee *et al.* 1990; Fang and Grumet 1990), where this method was successfully used.

Polymerase chain reaction (PCR)

The use of PCR analysis to confirm the presence of foreign DNA in transgenic plants has several advantages over Southern analysis. Southern hybridizations, while a reliable procedure to show the presence of foreign genes, are labour-intensive and difficult to perform on a large scale since they require large amounts of DNA and are time-consuming (Lassner *et al.* 1989). In contrast, detection of the foreign genes is considerably quicker using PCR analysis, and the procedure requires a minimal amount of DNA (20 ng) (Hamill *et al.* 1990).

The PCR cycle is relatively simple and consists of three major steps. The reaction requires a single-stranded template; therefore, the first step requires high temperatures to denature the double-stranded DNA. Subsequent cooling allows two specific oligonucleotide primers (which are complementary to the sequences flanking the target region) to anneal to the single-stranded template DNA. These primers are necessary for the initiation of the third step, which involves extension via DNA polymerase (Saiki 1990; Van Brunt 1990). This reaction is cycled by sequentially raising and lowering the incubation temperatures. After each amplification cycle, the number of copies of a specific DNA sequence is doubled since each newly synthesized DNA segment serves as a template for the next cycle, and the result is an exponential accumulation of the specific target fragment (Saiki 1990; Van Brunt 1990). Several oligo-nucleotide primer sequences have been synthesized for the detection and amplification of specific sequences in transformed plant tissues, including the NPT II gene, GUS gene, and NOS gene (Chee et al. 1989; Hamill *et al.* 1990). In all cases, only plants which contain the foreign gene yield the expected amplification band.

Western blot analysis

Western blotting is similar to Southern blotting with one exception being that proteins are used instead of DNA. For this analysis, proteins are isolated from transgenic tissues and are solubilized with detergents. Many methods have been described to separate native and denatured proteins, but the most widely used technique is SDS polyacrylamide gel electrophoresis (SDS-PAGE). With SDS-PAGE, proteins in a complex mixture are separated on the basis of molecular weight. The proteins are then transferred from the gel to a solid support (usually a nitrocellulose filter) which may then be stained. The filter is subsequently exposed to antibodies specific for the target protein. Finally, the antibody-protein complex is detected by secondary immunological reagents which produce a colorimetric reaction (Sambrook *et al.* 1989).

This technique is widely employed to detect proteins in transgenic plants, such as chitinases (Linthorst *et al.* 1990; Neuhaus *et al.* 1991; Nielsen *et al.* 1993).

General approaches to genetic engineering of plants for fungal disease resistance

Plants exhibit natural resistance to fungal attack. Classical genetic studies have shown that resistance is commonly conferred by a single gene, termed an R-gene, with the resistance allele usually dominant to the susceptibility allele (Bennetzen 1984; Ellis *et al.* 1988). Similarly, when races of the pathogen which differ in their reaction (virulence or avirulence) to a particular host plant are inoculated, studies have shown that a single gene may be responsible for the difference, with avirulence usually being dominant to virulence. These findings have led to the 'gene-for-gene' concept (Cramer and

Radin 1990; Ellis *et al.* 1988; Lamb *et al.* 1992), in which incompatibility results

from an interaction between any matched pair of dominant gene products.

Some of these R-genes have been isolated and cloned from plants and include

pathogenesis-related (PR) proteins, as well as ribosome inactivating proteins.

In addition to the single gene defense mechanism, the plant defense

arsenal also comprises a variety of responses which require the action of many

genes. Some of these responses include the production of phytoalexins,

phenolics, lignins, etc.

It is therefore conceivable that the introduction of genes which encode

proteins with demonstrated antifungal activity into the plant genome would

increase the defense response to fungal attack. Examples of successful

engineering of these genes into plants and their response to infection are few,

and are described in more detail below.

Pathogenesis-related proteins

The challenge of plants by potentially pathogenic organisms, and by

various other stresses, has been shown to result in the accumulation of PR-

proteins (Broglie *et al.* 1993; Lamb *et al.* 1992). These PR-proteins have been

found in most plant species studied and they have been separated into five

families or groups (Stintzi *et al.* 1993). PR-proteins are monomers of low

molecular mass (8-50 kDa) and may be localized in the vacuolar compartment or

the intercellular spaces near the cell wall (Stintzi *et al.* 1993). These proteins are

stable at low pH and are relatively resistant to proteases of endogenous or

exogenous origin (Stintzi *et al.* 1993). Some members of the different PR

families have also been found to be induced by polyacrylic acid, amino acid

derivatives, heavy metal salts, salicylic acid, air pollutants, and phytohormones

such as ethylene (Punja and Zhang 1993; Stintzi *et al.* 1993).

It is conceivable that enhancement of PR-protein production through constitutive expression in transgenic plants could render the plant more tolerant to a broad range of pathogens. However, results from constitutive expression of single PR-protein genes in transgenic plants have not been conclusive.

The PR-1 group were first discovered in response to virus infection, and members of this group were the first to be purified. Recent reports suggest that some PR-1 proteins have antifungal activity *in vitro* against *Phytophthora infestans* (Stintzi *et al.* 1993). Recently, genetic engineering studies to produce transgenic tobacco plants expressing high levels of PR-1a in a constitutive manner, showed significantly more tolerance to infection by *Peronospora tabacina*, the causal agent of blue mold disease of tobacco (Stintzi *et al.* 1993).

The hydrolytic enzymes, B-1,3-glucanase and chitinase, are encoded by the PR-2 and PR-3 gene families, respectively (Stintzi *et al.* 1993). A large number of these enzymes have been purified and characterized (Stintzi *et al.* 1993). These PR-proteins are usually monomers with a molecular mass in the 25-35 kDa range, with most having endolytic activity.

B-1,3-glucanase produces oligomers of chain lengths of two to six glucose units from 1,3-B-glucans, such as laminarin. Chitinase hydrolyses the chitin polymer at B-1,4-linkages to release *N*-acetylglucosamine oligomers (Collinge *et al.* 1993; Punja and Zhang 1993). Chitinase has no known substrate in the plant itself, and the substrate for B-1,3-glucanase, callose, is usually present only in small quantities (Mauch *et al.* 1988). However, chitin and B-1,3-glucans are major structural components of the cell wall of many phytopathogenic fungi, suggesting that these enzymes could play a role in inhibiting fungal growth and invasion within the plant (Punja and Zhang 1993; Stintzi *et al.* 1993). Antifungal activities of these enzymes have been

demonstrated *in vitro* against many fungi, causing lysis of hyphal tips (Collinge *et al.* 1993; Mauch *et al.* 1988).

Glucanases have also been engineered into transgenic plants, but no increase in disease tolerance has yet been reported (Lamb *et al.* 1992). However, glucanases and chitinases together have been shown to act synergistically to fungi *in vitro* (Mauch *et al.* 1988). Recently, a study in which tomato plants were simultaneously engineered with genes coding for a class I chitinase and class I B-1,3-glucanase, showed increased resistance to infection by *Fusarium oxysporum* f.sp. *lycopersici* (Melchers *et al.* 1993).

Recently, a novel family of PR genes, SAR-8.2, was discovered from virus-infected tobacco plants. Transgenic tobacco plants expressing a chimeric SAR-8.2 cDNA were reported to be highly resistant to *Phytophthora parasitica* var. *nicotianae* (Kamoun and Kado 1993). However, the basis for increased resistance in these plants is not known at present.

Ribosome-inactivating proteins

The seeds of several cereal plants are known to contain proteins which are toxic to some pathogens. Barley seeds contain ribosome-inactivating proteins (RIP) which, like the wheat homologue tritin and the related ricin A-chain of castor bean, inhibit protein synthesis by specific RNA *N*-glycosidase modification of 28S rRNA (Lamb *et al.* 1992; Logemann *et al.* 1992). RIPs are not toxic to plants, but show varying degrees of activity to a number of plant pathogenic fungi *in vitro* (Logemann and Schell 1993). Expression of the barley RIP cDNA under the control of a potato wound-inducible (*wun1*) promoter in transgenic tobacco plants conferred increased protection against *Rhizoctonia solani* (Logemann *et al.* 1992). RIPs have been shown to interact synergistically with chitinases *in vitro*; therefore, current experiments aim to test whether the

expression of both proteins may confer still higher levels of fungal protection in transgenic plants (Lamb *et al.* 1992; Logemann *et al.* 1992).

Phytoalexins

Phytoalexins are toxic compounds synthesized by plants in response to microbial infection and other stresses (VanEtten *et al.* 1989). These low molecular weight compounds, like PR proteins, are also triggered by mechanical injury, ultraviolet irradiation, and a variety of chemical elicitors (Harms 1992). Phytoalexins are part of the localized hypersensitive response at the site of pathogen entry or at the site of damage, which involves cell trauma and death (VanEtten *et al.* 1989). Because phytoalexins have significant antimicrobial activity against various fungal and bacterial pathogens, methods to enhance or modify their production *in planta* are currently being investigated. Since the pathways are complex and involve a multitude of genes, progress to date has been slow.

Compatible pathogens have evolved mechanisms to overcome the inhibitory activity of phytoalexins, such as through detoxification and/or toleration (VanEtten *et al.* 1989). Therefore, strategies to engineer plants with these defensive mechanisms may also disrupt the infection process.

Pathogens are often less tolerant to phytoalexins of non-host plant species; therefore, it has been suggested that if the phytoalexin biosynthetic pathway in a host plant were altered so that different phytoalexins were produced, the pathogen may not be able to detoxify or tolerate them (VanEtten *et al.* 1989). Since different plant species produce different phytoalexins, the interspecies transfer of biosynthetic genes could provide the basis for engineering novel classes of phytoalexins in crop plants. Confrontation of pathogens with new phytoalexins could potentially be an attractive method for

enhancing disease resistance (Harms 1992). The biosynthetic pathway leading to phytoalexin production has been studied in detail and considerable information of the steps involved is now available (Harms 1992; VanEtten *et al.* 1989), and effective alterations of these pathways may soon be feasible. Such alterations could be achieved, for example, by introducing genes encoding for enzymes that provide additional steps to the existing pathways (VanEtten *et al.* 1989). However, this approach requires improved knowledge of the regulation of the signal transduction pathways leading to phytoalexin accumulation and on the potential effects of expressing secondary metabolites in the transgenic plants.

Toxin-degrading enzymes

It is known that some pathogen toxins can cause severe symptoms on plants and often contribute to the virulence or the pathogenicity of the invading pathogen (Yoneyama and Anzai 1993). The toxins can be classified into two categories, host-specific and non-host specific. Host-specific toxins are those which are toxic only to the hosts of the pathogen, with the potential virulence of the pathogen being dependent on the amounts of toxin produced, and showing little to no toxicity to nonsusceptible plants. In contrast, non-host specific toxins produce symptoms not only on the host plant but also on other species of plants not normally attacked by the pathogen in nature (Agrios 1988). To date, several of these toxins have been isolated and identified from phytopathogenic fungi. Most of the host-specific toxins isolated so far are produced by species of *Alternaria* and *Helminthosporium*, and non-host specific toxins are produced by fungi such as *Fusarium oxysporum* and *Helminthosporium sativum*. To confer resistance in plants to these compounds, one approach utilizing genetic engineering would be to degrade or inactivate the toxin. If the detoxifying gene

of the pathogen toxin were introduced into the host plant, the genetically engineered plant might be protected against the disease. To date, there have been no reports of transgenic plants expressing fungal detoxifying genes. However, a similar strategy has been reported in which a gene encoding a tabtoxin-detoxifying enzyme derived from *Pseudomonas syringae* pv. *tabaci* was introduced into tobacco plants. The transgenic plants were found to not only exhibit high resistance to tabtoxin, but also became resistant to infection by *P. syringae* pv. *tabaci* which causes wildfire disease (Yoneyama and Anzai 1993).

Fusaric acid (FA) is produced by several species of *Fusarium*, which are often wilt-inducing vascular pathogens (Agrios 1988). Recently, FA-detoxifying genes have been identified, cloned, and sequenced from a bacterium (Kamoun and Kado 1993) and attempts to express them in transgenic plants are underway.

Chitinases

Plant chitinases have been characterized and grouped into at least four classes based on their primary structure (Collinge *et al.* 1993; Flach *et al.* 1992; Punja and Zhang 1993). Class I chitinases comprise the majority of the chitinases described. They are enzymes which have a basic isoelectric point and contain an amino-terminal cysteine-rich domain of approximately 40 amino acids which is thought to be involved in chitin binding (Flach *et al.* 1992; Collinge *et al.* 1993; Punja and Zhang 1993). A glycine- and proline-rich region separates the cysteine-rich domain from the catalytic domain. A C-terminal extension of seven amino acids has been shown to be involved in targeting the protein to the vacuole (Neuhaus *et al.* 1991). Class II chitinases lack the N-terminal cysteine-rich domain but have a high amino acid sequence identity to the main structure of class I chitinases (Collinge *et al.* 1993; Punja and Zhang

1993; Stintzi *et al.* 1993). These proteins are acidic and are located in the extracellular compartment (Flach *et al.* 1992; Punja and Zhang 1993). Class III chitinases show no sequence similarity to enzymes in classes I or II. These proteins can be acidic or basic, and seem to be compartmentalized in the extracellular space (Flach *et al.* 1992). Class IV chitinases contain a cysteine-rich domain and a conserved main structure similar to those of class I, but are smaller due to several deletions (Stintzi *et al.* 1993). Other chitinases which differ from the above have an acidic isoelectric point, are extracellular and contain a cysteine-rich domain. These enzymes have been found in bean, poplar and yam and may be a subclass of class I (Punja and Zhang 1993).

Genetic engineering of chitinases in plants

There are several reports of the successful introduction of chimeric chitinase genes into plants. The first report was the introduction of an exochitinase gene from *Serratia marcescens* into tobacco, which resulted in enhanced exochitinase activity (Nagel *et al.* 1990) and reduction in disease severity (Punja and Zhang 1993). The first report of enhanced antifungal activity, using genes of plant origin, in a transgenic plant was due to the introduction of a bean vacuolar chitinase gene (class I), under the control of the constitutive promoter of the cauliflower mosaic virus (CaMV) 35S, into tobacco and *Brassica napus* (Broglie *et al.* 1991). Expression of this protein in both plant species resulted in reduced fungal growth and delayed symptom development by *Rhizoctonia solani*, the causative agent of post-emergent damping-off (Broglie *et al.* 1991). In the bean plants, enhanced resistance appeared to be correlated with the level of bean chitinase expression; plants containing higher levels of the protein displayed a greater survival rate in fungal-infested soil (Broglie *et al.* 1993). In the transgenic canola plants infected with *R. solani*, a reduction in

fungal biomass was due to increased hyphal alterations leading to fungal lysis and chitin breakdown (Benhamou *et al.* 1993). The resistance observed in this study approached potentially useful levels with regard to reduced crop damage at inoculum densities likely to be encountered in the field (Broglie *et al.* 1993). There are also other reports of transgenic tobacco expressing either the tobacco class I chitinase, or the class III chitinases from tobacco (acidic) or from cucumber (acidic), in which significant resistance against *R. solani* was observed (Stintzi *et al.* 1993).

In contrast, preliminary infection experiments of transgenic *Nicotiana benthamiana* plants constitutively expressing an acidic class III chitinase gene from sugar beet (*Beta vulgaris*), did not show increased resistance to *Cercospora nicotianae* (Nielsen *et al.* 1993). Likewise, transfer of a tobacco basic vacuolar chitinase gene under the control of the CaMV 35S promoter into *Nicotiana sylvestris* showed unaltered sensitivity to *C. nicotianae*, even in plants expressing high levels of the protein (Lamb *et al.* 1992; Punja and Zhang 1993). However, in a different study using transgenic *Nicotiana sylvestris* plants expressing the CaMV 35S-vacuolar tobacco chitinase construct, the roots were colonized to a lesser extent by *R. solani* than control plants and had less reduction in fresh weight (Vierheilig *et al.* 1993). In this study, it was also found that expression of the transgenic protein did not interfere with colonization by the mycorrhizal symbiont *Glomus mosseae*. Other encouraging results have been shown with transgenic tomato plants constitutively expressing bacterial chitinases, which resulted in increased resistance to *Botrytis cinerea* infection (Bedbrook 1993).

Recent reports have suggested that the intracellular localization of basic chitinase may prevent its early involvement in defense against invading pathogens (Punja and Zhang 1993; Stintzi *et al.* 1993). Other reports have also

indicated that basic chitinases have greater antifungal activity than acidic forms of the enzyme (Melchers *et al.* 1993), and thus, attempts to target the basic chitinases to the extracellular spaces are in progress.

In conclusion, the above examples demonstrate the great potential of molecular biology and genetic engineering to increase protection of plants to microbial attack. The advantages of improved fungal resistance in crops include a reduction in application of chemicals, and an increase in agricultural productivity.

Objectives

Previous reports of *Agrobacterium*-mediated transformation of carrot have only demonstrated introduction of genes encoding for the neomycin phosphotransferase II enzyme or B-glucuronidase. Carrot is susceptible to a number of important fungal pathogens for which there is no genetic resistance. Thus, the introduction of an antifungal protein, such as chitinase, could provide an interesting study to not only demonstrate the introduction of a potentially agronomically important gene, but to also potentially enhance tolerance to pathogens. The objectives of this research project were:

1.   To construct and analyse binary vectors with genes encoding the enzyme chitinase, and to introduce them into infective *Agrobacterium* strains.

2.   To develop methodology to successfully transform carrot with *Agrobacterium* strains. Evaluate all possible parameters, including explant age, co-cultivation, selection and regeneration.

3.   To evaluate the use of PCR to confirm presence of transformants.

4.   To develop methodology to successfully conduct Southern Blot analyses.

Chapter II

Agrobacterium-mediated transformation of carrot (*Daucus carota* L.) with chitinase encoding genes

Introduction

Carrot (*Daucus carota* subsp. *sativa*) is an important horticultural crop which is grown worldwide (Ammirato 1986; Peterson and Simon 1986; Simon 1984). Genetic improvement of this crop has been achieved using methods in plant breeding (Ammirato 1986; Peterson and Simon 1986; Simon 1984), tissue culture techniques, such as anther culture (Ammirato 1986), utilization of somaclonal variation (Ammirato 1986; Sung and Dudits 1981), protoplast fusion (Sung and Dudits 1981) and direct DNA transformation (Bower and Birch 1993; Rasmussen and Rasmussen 1993; Dröge *et al.*1992). *Agrobacterium* -mediated transformation of carrot has been recently reported by a few researchers (Pawlicki *et al.* 1992; Scott and Draper 1987; Thomas *et al.* 1989 ; Wurtele and Bulka 1989). In these studies, the reporter gene B-glucuronidase (GUS), or the selectable marker gene neomycin phosphotransferase (NPT II) were introduced and expressed. In addition, there is one report of the expression of phosphinothricin-*N*-acetyltransferase conferring herbicide resistance (Dröge *et al.* 1992). The introduction of other potentially useful agronomic traits, such as tolerance to disease, has not been achieved in carrot using *Agrobacterium* transformation. The purpose of this research was to develop methodology to successfully transform carrot with various *Agrobacterium* strains to introduce genes coding for chitinases.

Chitinases are members of a group of proteins known as pathogenesis-related (PR) proteins. Their expression in plants is increased by pathogen attack and certain abiotic stresses (Punja and Zhang 1993; Samac and Shah 1991), and they accumulate intracellularly in the central vacuole (basic chitinases) or extracellularly in the intercellular space (acidic chitinases) (Broglie *et al.* 1991; Linthorst *et al.* 1990). Evidence for the role of chitinases in the defense response of plants is beginning to emerge (Broglie *et al.* 1991; Benhamou *et al.* 1993). Chitinases catalyze the hydrolysis of chitin, a substrate which is not found in plants but which is a component of the cell walls of many fungi (van Loon *et al.* 1987). Thus, exposure of fungal cells to chitinases has been shown to cause them to lyse (Mauch *et al.* 1988; Punja and Zhang 1993). To determine whether enhanced expression of chitinases in plants could also have a similar effect on fungi *in planta*, a series of experiments were initiated to introduce different chitinase encoding genes into carrot. These include chitinases recently cloned from petunia (Linthorst *et al.* 1990), tobacco (Linthorst *et al.* 1990), and bean (Broglie *et al.* 1991). Since carrot is naturally susceptible to a number of important fungal pathogens (Ammirato 1986; Peterson and Simon 1986; Strandberg 1993), the overexpression of this protein could lead to enhanced protection. In this study, the effects of *Agrobacterium* strain, plasmid, carrot cultivar, age of explant, and co-cultivation time on the frequency of transformation of carrot are described.

Materials and methods

Plant materials

Carrot seeds of cultivars Golden State, Danvers Half Long, Nantes Long, Scarlet Nantes and Nanco were disinfected by briefly washing in Liqui-Nax

detergent (Alconox, Inc. N.Y), followed by soaking in 70% ethanol for 30 s, then 2 min in a 5% solution of commercial bleach (Javex, 6.25% sodium hypochlorite), 2 min in a 1% solution of benzalkonium chloride 17% solution (Calbiochem Co., La Jolla, CA) (in 10% ethanol), and lastly rinsing three times in sterile distilled water (1 min each). The seeds were transferred to water agar plates containing 10 g L^{-1} glucose, 100 mg L^{-1} benomyl (as Benlate, 50% WP), 100 mg L^{-1} dichloran (as Botran, 75% WP), and 100 mg L^{-1} ampicillin and incubated at 23-28 °C. Once germinated (about 10-15 days), seedlings were transferred to Magenta boxes (Magenta Corp., Chicago, IL) containing approximately 30 mL of full-strength growth regulator-free MS medium (Murashige and Skoog 1962) with myo-inositol (100 mg L^{-1}), thiamine HCl (0.8 mg L^{-1}), 30 g L^{-1} sucrose, and 10 g L^{-1} tissue culture agar (Sigma). The pH of the medium was adjusted to 5.8 prior to autoclaving at 15 psi for 15 min. Sterile ampicillin was added at a concentration of 100 mg L^{-1} to prevent bacterial contamination. The seedlings were grown at 23-28 °C under cool-white fluorescent lamps (intensity of 450 μmol m^{-2} s^{-1}) with a 16 h light and 8 h dark cycle.

Tissue culture conditions

The medium used in callus induction and regeneration studies was full-strength MS medium containing thiamine, sucrose, agar and ampicillin at the concentrations specified above. The influence of 2,4-D concentration on callus growth and somatic embryogenesis was evaluated. The 2,4-D stock solution was filter-sterilized and was added to the medium after autoclaving to achieve a final concentration of 0.50, 2.0, or 4.5 μM. Epicotyls from two week-old aseptic seedlings (cv. Golden State and Nanco) were cut into approximately 1-cm-long segments and placed on the media. Approximately 8-10 explants were placed in

each petri dish (100 x 15 mm), with 4-6 replicate dishes per treatment. All plates were sealed with Parafilm® and incubated under the same conditions as above. The effect of cultivar on frequency of somatic embryogenesis was also evaluated using epicotyls from aseptic seedlings of the cultivars Nanco, Golden State and Danvers Half Long. Approximately 8-10 explants were placed on MS medium containing 4.5 µM 2,4-D, with 5-6 replicate dishes per cultivar.

Bacterial strains and plasmids

Disarmed *Agrobacterium tumefaciens* strains MOG 101 (octopine type) and EHA 105 (leucinopine, supervirulent type) (courtesy of MOGEN Int. nv, The Netherlands) were used. Each strain harboured one of three binary plasmids. Two plasmids, pMOG196 (14.0 kb) and pMOG198 (14.2 kb) (MOGEN Int. nv) (Fig. 1), contain between the T-DNA borders, a petunia acidic chitinase gene (Pach 1) or tobacco basic chitinase gene (Tbch 1), respectively, driven by the CaMV 35S promoter (Linthorst *et al.* 1990). A third plasmid, pGA492-CHN (14.8 Kb) (Fig. 2), was constructed by excising the 2.5 kb EcoR I-Cla I fragment of pk35CHN641 (Broglie *et al.* 1991), consisting of the bean endochitinase (CH5B) coding region fused to the CaMV 35S promoter, ligating into a EcoR I-Cla I linearized plasmid pGA492 (An 1987) and cloned in *Escherichia coli* DH5α. Plasmid pGA492-CHN was mobilized from *E. coli* into *A. tumefaciens* strains MOG101 and EHA105 via triparental matings (L. Melchers, MOGEN Int. nv, *pers. comm.*) (see Appendix). All three plasmids also contained a transferable selectable marker for kanamycin resistance (the neomycin phosphotransferase gene, NPT II, driven by the nopaline synthase (NOS) promoter). A single colony of each of the *Agrobacterium* strains was inoculated into 50 mL of Luria broth (Sambrook *et al.*, 1989) medium (with kanamycin 100 mg L^{-1}) and grown overnight at 29 °C on a rotary shaker at 200 rpm. Each culture was then diluted

Figure 1. Schematic diagram of the binary vectors pMOG 196 and pMOG 198. These vectors constructed by Linthorst *et al.* (1990) were used to introduce an acidic petunia chitinase and a basic chitinase gene, respectively. The vectors contain an expression cassette consisting of the cauliflower mosaic virus 35S promoter, and the nopaline synthase transcription terminator.
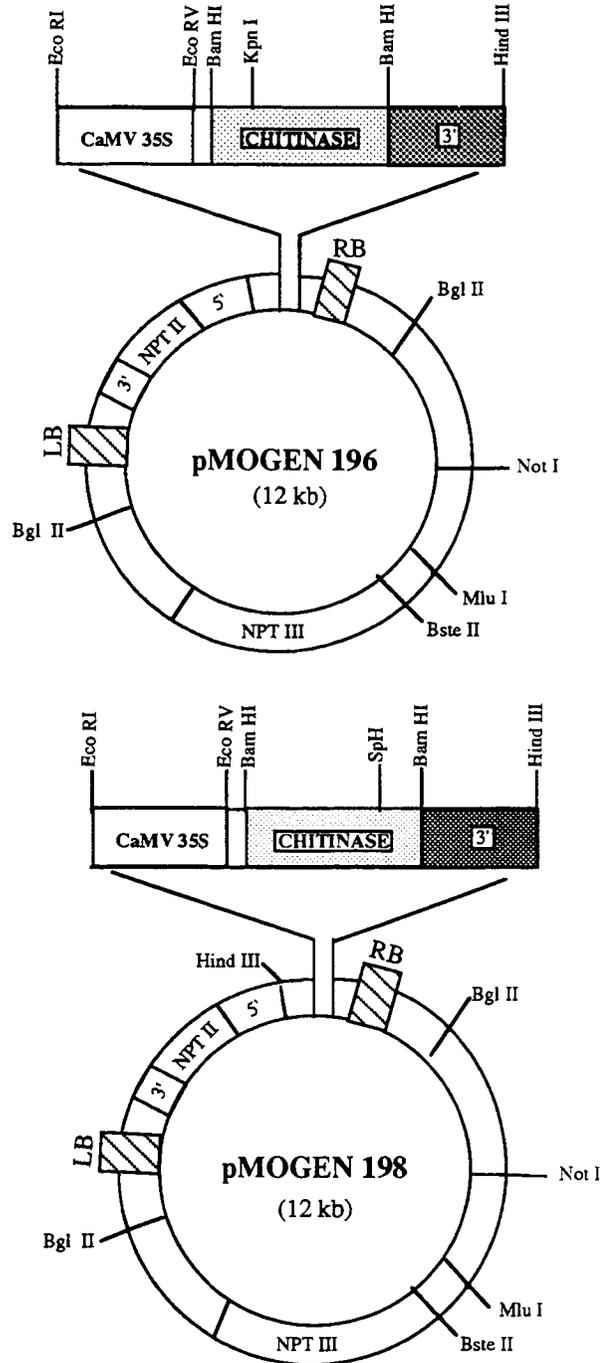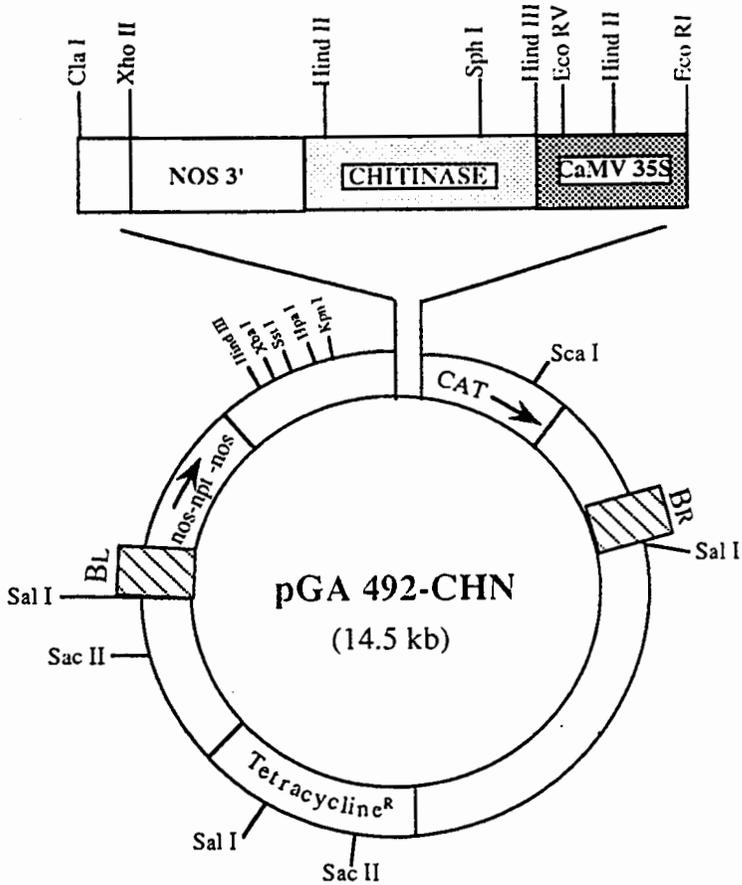
Figure 2. Schematic diagram of binary vector pGA492-CHN. This vector was constructed by ligating the 2.5 kb EcoR I-Cla I fragment of pk35CHN641 (Broglie *et al.* 1991), into linearized plasmid pGA492 (An 1987). The ligated fragment consists of the bean chitinase gene (CH5B) fused to the CaMV 35S promoter and NOS terminator.

1:50 in Minimal Medium (MM) (Turk *et al.* 1991) (with kanamycin 100 mg L^{-1})

and grown overnight under the same conditions. The bacterial suspensions

were then diluted in MM (without kanamycin) to a density of A_{600}= 0.15, placed

at 29 °C at 200 rpm, and allowed to grow to a density of A_{600}= 0.25-0.30.

Aliquots (15 mL) of each of the cultures were centrifuged at 3,700 x g for 5 min

at ambient room temperature. The supernatant was discarded and the pellets

were resuspended in MS medium (pH 5.3 when using EHA105 and pH 5.8 when

using MOG101) containing 100 µM acetosyringone and diluted to a final density

of A_{600}= 0.05 (approximately 1 x 10^8 cells mL^{-1}) (Turk *et al.* 1991).

Transformation Procedure

Epicotyl explants (1-cm-long) from 2-5 week-old aseptic carrot seedlings

were precultured for 2 days on MS medium supplemented with 4.5 µM 2,4-D

prior to the transformation experiments. After the preculture period, the explants

were immersed in 10-25 mL of the bacterial suspension for 4 min, rinsed in liquid

MS medium, dried briefly on sterile filter paper, and placed back on preculture

medium. Explants were co-cultivated in the dark for 2-4 days at 26 °C. The

infected explants were subsequently rinsed in sterile water, blotted dry, and

placed on selective medium (MS medium containing 4.5 µM 2,4-D, 400 mg L^{-1}

carbenicillin, and 25 mg L^{-1} kanamycin). The tissue culture plates (60 x 15mm),

containing approximately 8-10 explants each, were incubated in the dark at an

ambient temperature of 23-28 °C. Both positive and negative controls were

included in the experiments. The positive control consisted of non-cocultivated

explants cultured on antibiotic-free regeneration media (MS containing 4.5 µM

2,4-D), and the negative controls consisted of non-cocultivated explants cultured

on selective medium. After 4 weeks, all explants were transferred to fresh

selective medium with a higher concentration of kanamycin (100 mg L^{-1}) and

and placed under cool-white fluorescent lamps with a 16-h light and 8-h dark cycle at ambient temperature. Calli were subcultured to fresh selective medium every 4-6 weeks, over a 4-6 month period. Upon production of somatic embryos, these calli were either placed into 50 ml of liquid MS medium containing 0.5 µM 2,4-D and 50 mg L^{-1} of kanamycin or subcultured directly onto MS medium lacking plant growth regulators or kanamycin (MSO). Suspension cultures (in 50 mL of medium, incubated at 150 rpm) were subcultured to fresh medium every 2 weeks. Once the suspension cultures were established, approximately 0.5 mL of each suspension was spotted onto MSO medium and allowed to grow for 4-6 weeks. When plantlets were produced, they were transferred to MS medium containing 50 mg L^{-1} kanamycin and no 2,4-D. Within 4 weeks, rooted plantlets were transferred into sterile soil and placed into a growth chamber maintained at 26 °C, 85% RH and a 16-h photoperiod.

Data analysis

The data on percentage of somatic embryo production following different treatments from the replications and repetitions were pooled together and analyzed for significant differences using Chi-square analysis ($P \leq 0.05$). In addition, the standard deviation was considered when comparing means from different treatments. In all experiments which evaluated each of the parameters influencing *Agrobacterium* transformation, the minimum number of explants included was 33; the maximum was 404. All experiments were repeated at least once; some were repeated 4-5 times over a two-year period.

PCR

Total nucleic acids were isolated from leaf tissue (1-2 g fresh weight) of transgenic and non-transgenic plants according to the protocol described by

Kanazawa and Tsutsumi (1992) and were subsequently treated with 50 µg mL^{-1} of RNAse. The DNA was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and ethanol-precipitated. Two specific sequences of the NPT II coding region (courtesy of Dr. M.M. Moloney, University of Calgary, Alberta) were used for PCR amplification of this gene in the genomic DNA. Oligomer A, a 17mer with 5'-3' sequence GATGGATTGCACGCAGG, was located 15 bp upstream from the start codon, and oligomer B, a 17mer with 5'-3' (bottom strand) sequence GAAGGCGATAGAAGGCG shared identity with the 3' region of the NPT II gene, 17 bp 5' of the stop codon. These primers amplified a region about 800 bp in size. Each PCR reaction (25 µL overlaid by 50 µL of mineral oil) consisted of 1X Taq buffer (MgCl$_2$-free), 2.5 mM MgCl$_2$, 200 µM of dNTPs, 0.5 µM of each oligo-nucleotide primer, and approximately 20 ng of template DNA. Each PCR reaction was incubated at 92 °C for 5-10 min and then 1.25 units of Taq polymerase (Promega) was added and the mixture quickly placed on ice. The temperature cycling for the PCR was conducted as follows: 29 cycles at 94 °C for 1 min, 54 °C for 2 min and 72 °C for 3 min. The 30th cycle was the same with the exception that DNA synthesis at 72 °C was carried out for 10 min. The PCR products were then analysed by electrophoresis on 2.0% agarose gels. The oligomers were also tested by amplifying the characteristic 800 bp region of the NPT II gene by using 10 ng of total *Agrobacterium* DNA containing pMOG196 as positive control template.

Southern hybridization analysis

Approximately 12 µg of total genomic DNA was digested with the restriction enzyme Hind III (BRL) according to manufacturer's specifications, and electrophoresed on a 0.7% agarose gel. DNA fragments were blotted to positively-charged nylon membranes (Boehringer Mannheim) by capillary action

with 20 x SSC and fixed by UV crosslinking. Filters were hybridized against a Dig-UTP PCR-labeled probe. The probe was made by amplifying the 800 bp fragment containing the coding region of the NPT II gene using the PCR temperature cyclings and conditions specified above. However, for this reaction the dTTP concentration was changed to 17 µM and Dig-UTP (Boehringer Mannheim) was added to a final concentration of 8.5 µM (A. Lévesque, Agriculture Canada, *pers. comm.*). Hybridizations were conducted using about 15 ng of the labeled probe per ml of hybridization solution containing 2% blocking buffer (Boehringer Mannheim) and 50% formamide (redistilled nucleic acid grade, BRL) and incubated at 40-42 °C for at least 15 h. Washes and chemiluminescence detection of hybridized filters were done as per Boehringer Mannheim's instructions. The blots were exposed to X-ray films (Kodak OMAT-K) for 1-24 h.

Western blot analysis

Leaves of transgenic carrot plants were frozen in liquid nitrogen immediately after being collected, and finely ground into a powder using a mortar and pestle. All extractions were performed at 4 °C unless indicated otherwise. The powder was extracted with 0.1 M sodium citrate buffer, pH 5.0; the crude homogenate was filtered through four layers of Miracloth and centrifuged at 20,000 g for 30 min. The supernatant was put onto an ultrafiltration unit (Amicon YM10 filter) for protein concentration adjustment. Protein samples were separated by 15% SDS-PAGE and transferred to 0.45 µm nitrocellulose membranes with a Bio-Rad Trans-Blot semi-dry electrophoretic transfer cell (Cat. No. 170-3940) as described in the instruction manual. Western blots were performed following the procedure described in the Bio-Rad Immun-Blot GAR-AP assay kit (Cat. No. 170-6509) instruction manual. The antibody raised

against tobacco basic chitinase (provided by Dr. B. Fritig, Institut de Biologie Moleculaire des Plantes, France) was used at 1:2000 dilution.

Results

Tissue culture

A series of experiments was conducted to determine the optimum concentration of 2,4-D required for callus induction and somatic embryogenesis in two carrot cultivars. When epicotyl explants were placed on MS medium with 0.50, 2.0, or 4.5 µM 2,4-D, calli developed at different rates, but overall somatic embryo frequency was only affected in Nanco and was lowest at 4.5 µM 2,4-D. In Golden State, 2,4-D concentration had no effect (Table 1). In all treatments, after four weeks of incubation, calli appeared homogenous and friable, and frequently had somatic embryos and small shoots. The effect of cultivar on frequency of somatic embryogenesis was also determined by comparing the final percentage of embryogenic calli after 2-4 months of incubation on 4.5 µM 2,4-D. The cultivar Nanco had the highest frequency (81.4%), followed by Golden State (77.2%) and Danvers Half Long (55.3%).

Transformation procedure

Development of calli and plantlets

Following co-cultivation, epicotyl explants were placed on selective medium with 25 mg L^{-1} of kanamycin for 4 weeks. Subsequently, healthy calli were subcultured to fresh selective media containing 100 mg L^{-1} kanamycin. This two-step procedure was selected to minimize potential inhibition of regeneration by kanamycin (Jia *et al.* 1989; van Wordragen *et al.* 1992). Initially, co-cultivated and control explants expanded to about the same size during

Table. 1. Effect of 2,4-D concentration on somatic embryo production in cultivars Golden State and Nanco.

Carrot cultivar [a]	Embryogenic calli (%) following exposure to 2,4-D [b]		
	0.5 μM	2.0 μM	4.5 μM
Golden State	13.2 (38) [c]	10.3 (39)	14.7 (34)
Nanco	25.4 (59)	19.2 (52)	7.0 (57)

[a] Epicotyl segments (1-cm-long) from 2-week-old aseptic seedlings

[b] Percentage of calli out of the total plated which developed somatic embryos after 2-4 months in culture

[c] Numbers in parentheses refer to total number of explants plated.

selection on 25 mg L^{-1} kanamycin (Fig. 3A). At 100 mg L^{-1}, only the co-cultivated explants and positive controls developed additional callus (Fig. 3B), while the negative control did not increase in size and was bleached.

Effect of explant age

Transformation efficiency was calculated as the total number of co-cultivated explants which produced embryogenic calli on selective medium relative to the total number of co-cultivated explants originally plated on media containing 100 mg L^{-1} of kanamycin. Epicotyl explants from 2, 3, 4, and 5 week-old aseptic seedlings of the carrot cultivar Golden State were co-cultivated for 2-3 days with the supervirulent *Agrobacterium* strain EHA 105 (containing either pMOG 196 or pMOG198). The transformation frequency ranged from 5.4% to 8.6% for two to five week-old seedlings, respectively (Fig. 4). These different explant ages were found to be not statistically different (P = 0.91); therefore, in further experiments, explant age was not considered to be a variable which could affect transformation frequency.

Effect of Agrobacterium plasmid

To determine whether transformation efficiency was influenced by the *Agrobacterium* plasmid, two experiments were conducted. In the first experiment, explants of cultivar Golden State were co-cultivated for 2-3 days with *Agrobacterium* strain EHA 105 with pMOG196 or pMOG198. The second experiment consisted of explants of the cultivar Danvers Half Long co-cultivated for 3 days with *Agrobacterium* strain MOG 101 harbouring plasmids pMOG196, pMOG198, or pGA492-CHN. With Golden State, the transformation efficiency was 5.6% when pMOG196 was used, and 6.4% with pMOG198. With Danvers Half Long, transformation efficiency was 7.3% (pMOG196), 2.4% (pMOG198),

Figure 3. *Agrobacterium*-mediated transformation of epicotyl explants of carrot. (A) Kanamycin-resistant calli (cv. Golden State) co-cultivated with EHA 105 (pMOG198) after 4 weeks on selective medium (25 mg L^{-1} kanamycin). (B) Close-up of growing callus on selective medium. (C) Production of somatic embryos upon subculture of calli to auxin-free medium. (D) Development of somatic embryos into shoots 4-6 weeks after plating onto auxin-free medium. (E) Growth of untransformed (left) and transformed (right) plantlets in kanamycin-containing medium (50 mg L^{-1}). (F) Transgenic carrot plant at maturity (cv. Golden State).
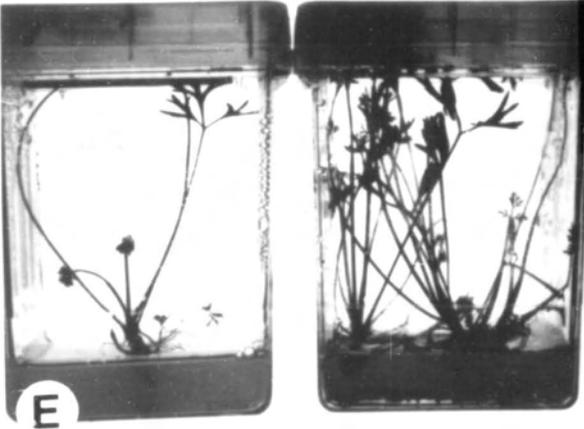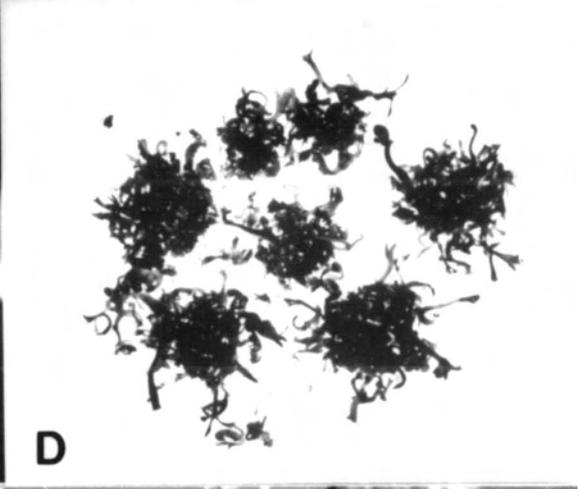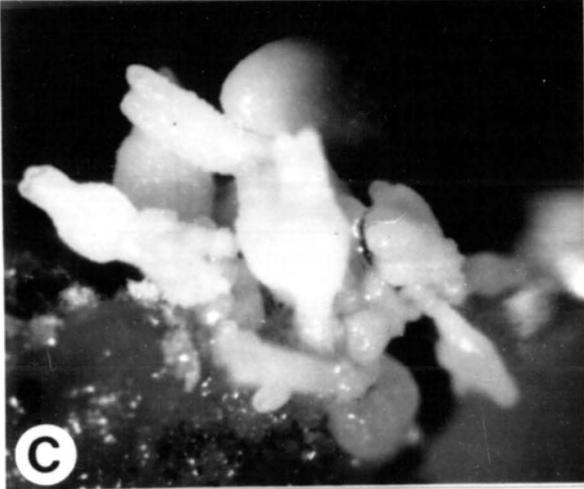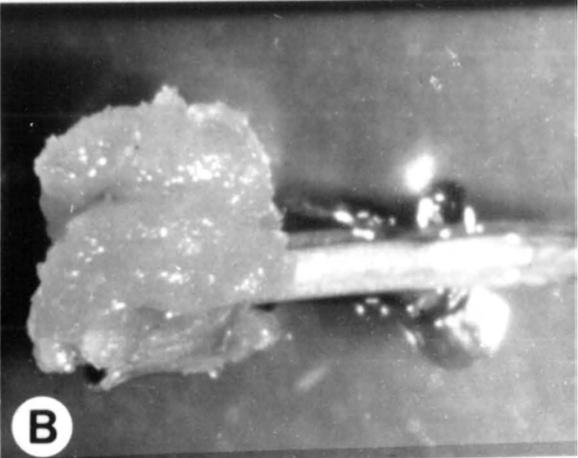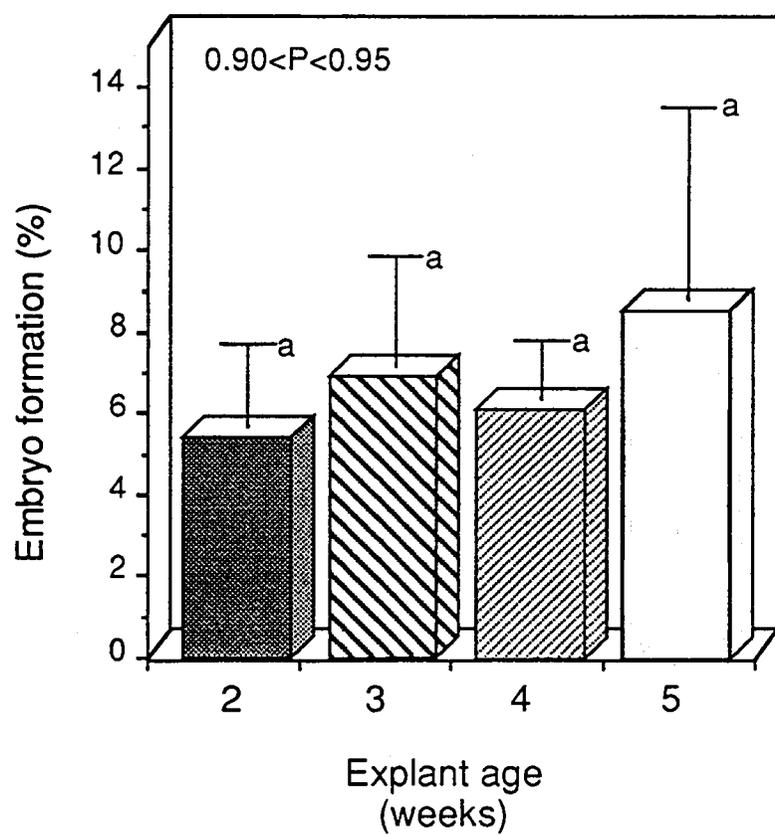
Figure 4. Effect of explant age on the transformation efficiency of carrot (cv. Golden State) co-cultivated with *A. tumefaciens* EHA 105 (pMOG196, pMOG198) for 2-3 days

and 1.0% (pGA492-CHN) (Fig. 5). Chi-square analysis (P≤ 0.05) showed that these transformation efficiencies were not significantly different.

Effect of co-cultivation period

Co-cultivation periods of 2, 3, and 4 days were tested. Epicotyl explants of cultivars Golden State and Danvers Half Long were co-cultivated for these periods following infection by *Agrobacterium* strain EHA 105 containing pMOG196 or pMOG198. The results showed that 2 or 3 days of co-cultivation yielded similar transformation frequencies, but these were significantly higher than a 4 day co-cultivation (Table 2).

Effect of carrot cultivar

Epicotyl explants of four carrot cultivars were co-cultivated for 2-3 days with the *Agrobacterium.* strain MOG101 (containing pMOG196, pMOG198, or pGA492-CHN). Scarlet Nantes had the highest transformation rate (6.2%), followed by Danvers Half Long (5.1%), Nanco (0.5%), and Nantes Long (0.0%). All cultivars were found to be significantly different (P = 0.05) (Fig. 6A). In a second experiment, cultivars Golden State, Nanco and Danvers Half Long were co-cultivated with *Agrobacterium* strain EHA 105 (containing pMOG196, pMOG198, or pGA492-CHN). Nanco had the highest transformation efficiency (12.1%), followed by Golden State (6.1%) and Danvers Half Long (1.8%) (Fig. 6B).

Effect of Agrobacterium strain

Explants of the cultivars Danvers Half Long and Nanco were co-cultivated for 3 days with *Agrobacterium* strains MOG 101 or EHA 105. The transformation efficiency was significantly affected by the *Agrobacterium* strain used (Table 3), with a marked strain-cultivar interaction observed.

48a

Figure 5. Effect of *Agrobacterium* plasmid on transformation efficiency of two carrot cultivars.
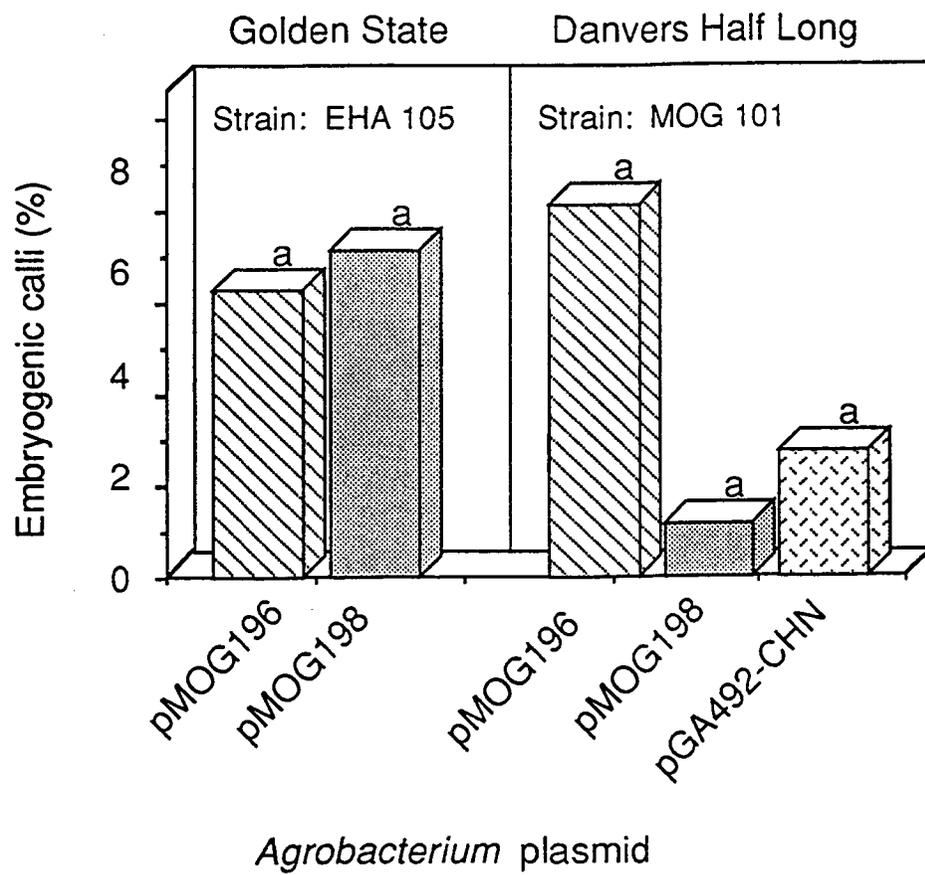
Table. 2. Effect of co-cultivation period of epicotyl explants of carrot with *Agrobacterium tumefaciens* strain EHA 105 on transformation frequency

Carrot cultivar [a]	Embryogenic calli (%) following a co-cultivation period of [b]		
	2 days	3 days	4 days
Golden State	6.5	6.0	0.0
Danvers Half Long	—[c]	1.8	0.0

[a] Epicotyl segments (1-cm-long) from aseptic seedlings

[b] Percentage of calli out of the total plated following co-cultivation which developed somatic embryos after 2-4 months in culture

[c] Not determined

49

Figure 6. Effect of carrot cultivar on transformation efficiency. Epicotyl

segments were taken from aseptic seedlings and co-cultivated for 2-3 days with

A. tumefaciens strain MOG 101 (A), or strain EHA 105 (B).
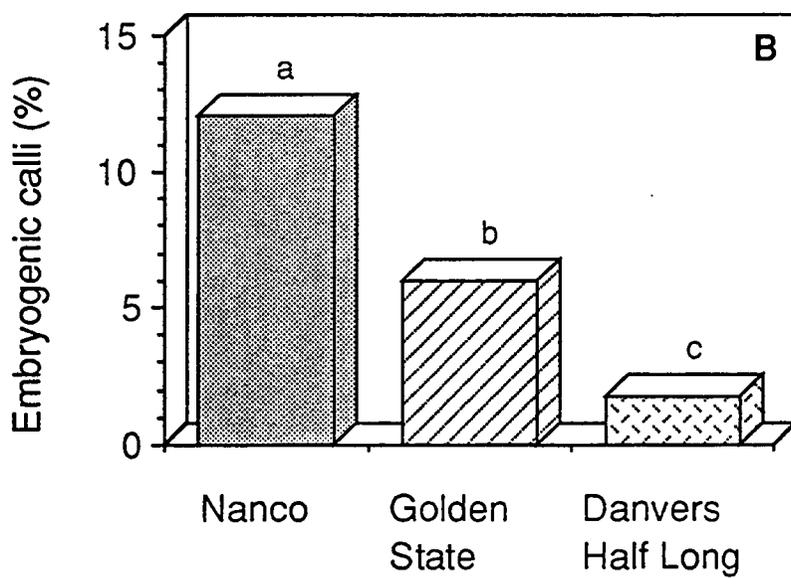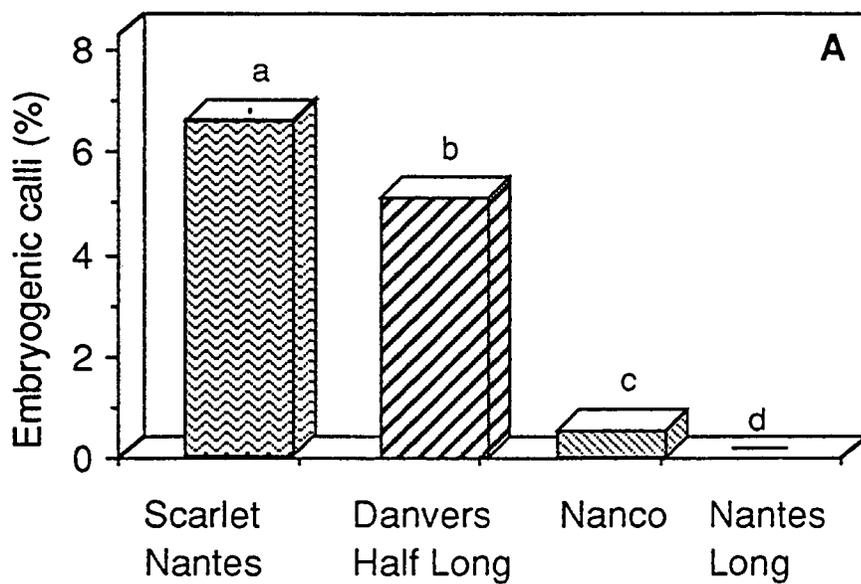
Table. 3. Effect of *Agrobacterium* strain on transformation frequency of carrot

| Carrot cultivar [a] | Embryogenic calli (%) following exposure to *Agrobacterium* strain [b] | |
	MOG 101	EHA 105
Danvers Half Long	5.1 (39) [c]	1.8 (386)
Nanco	0.5 (396)	12.1 (146)

[a] Epicotyl segments (1-cm-long) from 2-4 week-old aseptic seedlings

[b] Percentage of calli out of the total plated following co-cultivation which developed somatic embryos after 2-4 months in culture

[c] Numbers in parentheses refer to total number of explants plated. Data from the replications and repetitions was pooled.

Regeneration of transformed plants

Upon production of somatic embryos (Fig. 3C), calli were either placed into liquid MS medium (0.5 µM 2,4-D, 50 mg L^{-1} kanamycin) or subcultured onto MS medium without growth regulators or kanamycin (MSO). Once suspension cultures became established, approximately 0.5 mL of each suspension was spotted onto MSO. Within 4-6 weeks, these somatic embryos developed into shoots (Fig. 3D) and were transferred to MS medium containing 50 mg L^{-1} kanamycin. Plantlets which rooted and developed leaves were selected (Fig. 3E). These plants were transferred to soil and placed in a growth chamber, where they grew into mature plants (Fig. 3F).

PCR analysis

PCR amplification of the NPT II coding region was conducted with cultivar Golden State transformed with strain EHA105 containing pMOG198. The expected 800 pb band was obtained which was visible by exposing the ethidium bromide stained gel to UV light (Fig. 7), while control plants did not yield this band. Among all of the plantlets recovered through the tissue culture process, none were found which did not contain the NPT II insert (data not shown).

Southern hybridization analysis

Transformed plants of Golden State transformed with *Agrobacterium* strain EHA 105 (pMOG198) were analysed. Since the DNA isolated was readily digested by restriction endonucleases, it did not require further purification. Hind III digestion of DNA from two transformed plants, originating from one callus, yielded a single 6.3 kb fragment that hybridized with the NPT II probe, while no

53a

Figure 7. Results from polymerase chain reaction (PCR) amplification using primers specific to the NPT II gene coding region. Control plant lane contains 20 ng of non-transformed plant DNA. Vector DNA lane contains 10 ng of pMOG 196, positive control. Other two lanes contain 20 ng of DNA isolated from transformed plants number 1 and 21. DNA size standard (100 bp ladder, BRL) is shown.

800 bp –

hybridization was detected in the control plant DNA (Fig. 8). DNA from
pMOG198 was used as positive control.

Western blot analysis

Transgenic plants analysed did not contain chtinase protein at a level detectable
by Western blot analysis.

55a

Figure 8. Southern blot analysis of HindIII restricted DNA (12 µg/lane) from two transgenic carrot plants (plants 1 and 21) derived from strain EHA 105 (pMOG198). Filter was hybridized against a 800 bp Dig-UTP labelled fragment containing the NPT II coding region. Blot was exposed to X-ray film for 1 h following chemiluminescent detection.

1 21

6.3 Kb—

Discussion

I have successfully demonstrated transformation of carrot with two *Agrobacterium tumefaciens* strains and the introduction of both acidic and basic chitinase genes. Furthermore, the factors that can influence frequency of transformation have been described. While this research was in progress, a report on transformation of carrot with *Agrobacterium* strain C58C1 and expression of the NPT II gene was published (Pawlicki *et al.* 1992). Since my work has utilized different *Agrobacterium* strains and plasmids to those described (Pawlicki *et al.* 1992), it was necessary to optimize the various parameters influencing transformation with these strains.

Optimal callus induction, somatic embryogenesis and plantlet regeneration in different carrot cultivars in this study was obtained when epicotyl explants were placed on MS medium containing 0.5 µM of 2,4-D. However, it was important to omit the addition of 2,4-D once somatic embryos were formed to regenerate normal-appearing shoots. Similar observations have been reported by several researchers (Ammirato 1986; LoSchiavo *et al.* 1991; Zimmerman 1993). Upon transfer to auxin-free medium, the calli developed plantlets at a high frequency and could be maintained and grown to maturity. Our previous studies showed epicotyl explants to have a higher frequency of somatic embryos than cotyledon or leaf explants (data not shown). Pawlicki *et al.* (1992) obtained the same results when comparing epicotyl explants to hypocotyl and cotyledon explants. The frequency of somatic embryogenesis was also found to be cultivar dependent in our study. Similar findings have been made in other research (Feirer and Simon 1991; Krul 1993). These differences have been attributed to production of ethylene and to "embryo-specific" proteins in different carrot cultivars (Feirer and Simon 1991).

The frequency of transformation in the present study was also influenced by carrot cultivar, and ranged from 0% to 12.1%, with Nanco having the highest transformation frequency using strain EHA 105. These rates are lower than those obtained by Pawlicki *et al.* (1992) (46.7%), but are higher than those reported by Thomas *et al.* (1989) (5.8%). In these previous studies, transformation efficiency was expressed as the total number of embryogenic calli growing on selective medium (Pawlicki *et al.* 1992) or as the total number of kanamycin-resistant calli (Thomas *et al.* 1989). These differences may be also be attributed to the different *A. tumefaciens* strains used and to the binary plasmids harboured within them. A significant cultivar-strain interaction was observed in this study. Somatic embryogenesis in the positive controls was highest with Nanco followed by Golden State, Danvers Half Long, Nantes Long and Scarlet Nantes (data not shown). Upon co-cultivation, this order varied depending on the *Agrobacterium* strain used.

Age of epicotyl explants (from 2 to 5 weeks old) was not found to influence transformation efficiency in carrot. Although Pawlicki *et al.* (1992) reported that transformation rates were higher using explants from 3 and 4 week-old seedlings, and were lower with 1 and 2 week-old seedlings, explant age from these seedlings are comparable to our seedling ages (3 to 6 weeks old). Thus, explant ages of 3-4 weeks appear to be optimal.

Period of co-cultivation is known to influence the transformation process in several plant species (De Kathen and Jacobsen 1990; Fang and Grumet 1990; Jia *et al.* 1989; Torres *et al.* 1993) including carrot (Pawlicki *et al.* 1992). Although the transformation frequency following 2 and 3 days of co-cultivation was found to be similar, it was reduced significantly with a 4 day co-cultivation or after 7 days (Pawlicki *et al.* 1992). This reduction was attributed to excessive colonization of the explants by bacteria, followed by death.

The *Agrobacterium* plasmids did not appear to influence transformation efficiency in this study. However, since the three plasmids were similar in size, it is not known whether transformation would vary with different sized plasmids.

The strain EHA 105 (supervirulent) yielded higher transformation in Nanco than MOG 101 (nopaline). The reverse was observed with Danvers Half Long. Nopaline strains are often found to be less effective than supervirulent strains (Godwin *et al.* 1991; Komari 1989; Puonti-Kaerlas *et al.* 1989). There was also a cultivar-strain interaction, comparable to the host-strain interaction observed in other plant species (Godwin *et al.* 1991; Komari 1989; Puonti-Kaerlas *et al.* 1989). The basis for this interaction in carrot is not known.

In Southern blot analysis, digestion with Hind III, which cuts at the 5' end of the NPT II promoter (NOS), should yield a hybridizing fragment indicative of the chromosomal location of the inserted fragment within the carrot genome. Digestion with Hind III yielded a hybridizing band of about 6.3 Kb in size in two plants. The identical band size confirmed that both plants were derived from a single transformation event (one callus), and also provided evidence that the NPT II region of pMOG 198 had been integrated into the plant genome. The single hybridizing band indicated that one T-DNA copy was present in the genome of these transgenic plants.

Expression of the chitinase gene was analysed by Western blots. Preliminary results showed no detectable chitinase protein. It has been reported that the differential expression or the lack of expression of a gene can be attributed to 'positional effects' (Nehra *et al.* 1990; Hooykaas *et al.* 1992). Even though the chitinase genes contained their own constitutive expression signals, expression may still be influenced by the neighbouring chromosomal sequences. DNA methylation has been shown to be associated with the regulation of gene expression in a number of plant species (Meyer 1993). Therefore, integration of

a transgene into a hypermethylated region can cause the gene to also become methylated and be rendered transcriptionally inactive (Meyer 1993). Recently, it was reported that an increased degree of nonspecific genomic DNA methylation can occur, especially during *in vitro* culture of rhizogenic carrot callus tissue (Arnholdt-Schmitt 1993). It could be that the transformed carrot explants underwent similar methylation changes which inactivated the introduced chitinase genes. Bochardt *et al.* (1992) found that a silent GUS gene in tobacco could be reactivated by treatment with 5-azacytidine, a DNA methylation inhibitor. Therefore, use of this inhibitor in this study would either prove or disprove the role of methylation in the repression of chitinase gene expression.

A second possibility for the non-detectable chitinase protein is the phenomenon known as "co-suppression". Co-suppression is "the discovery that transferring a gene or gene fragment in the sense orientation back into a plant can result in the inhibition of expression of both the transgene and the endogenous gene from which it was derived" (Fray and Grierson 1993). The phenomenom is manifested by reduced levels of mRNA from the introduced transgene and , in many cases, of the endogenous gene (and closely related sequences) (Fray and Grierson 1993). Many examples of co-suppression in plants have now been reported (Brusslan *et al.* 1993; Fray and Grierson 1993; Hart *et al.* 1992;Napoli *et al.* 1990), although the mechanism is not known. Silencing of basic chitinase expression was also observed by Hart *et al.* (1992) in three independent lines of transgenic *Nicotiana sylvestris* . The results obtained by that group showed that about 25% of the plants exhibited the silent phenotype. Measurements of chitinase and chitinase mRNA in transgenic and control plants established that silencing resulted from co-suppression. A second observation, was that chitinase silencing was strongly influenced by the environment, since it occurred at high frequencies (20%-40%) in plants grown as

seedlings in closed cultured vessels (Hart *et al.* 1992). Therefore, it may be that since all of the carrot plants used in this study were grown in closed culture vessels that co-suppression could explain the non-detectable chitinase protein. However, the background enzyme levels were not measured.

Chimeric genes with the Cauliflower Mosaic Virus (CaMV) 35S RNA expression signals usually confer constitutive expression of the introduced gene. However, with this promoter, the levels of expression have been found to vary widely in different plant organs (Broglie *et al.* 1991; Higgins and Spencer 1991). Production of pea vicilin protein in transgenic tobacco was higher in younger leaves than in older leaves (Higgins and Spencer 1991). In the present study, carrot proteins used for Western blot analysis were obtained from leaves of plants which were about 10 months old. Therefore, use of younger plants may have resulted in high amounts of the chitinase protein. Broglie *et al.* (1991) studying the expression of a chimeric CaMV 35S-bean chitinase gene in tobacco found higher amounts of the chitinase protein in roots than in leaves. However, since roots were not assayed in this study, it is not known if this would also be the case.

In this study, since only one line of transgenic carrot plants was studied, it is difficult to ascertain the exact reason why the chitinase protein was not present at detectable levels. However, additional independent lines of transformants are currently being produced which will allow for a more indepth analysis. In plants not producing the chitinase protein, these analyses could aid in determining the level at which protein synthesis was being suppressed. These analyses could include the development of chitinase probes to be used in Southern blots. Excision of the chitinase gene in the transgenic plants would allow us to determine if any rearrangements of the gene had occurred during insertion that could lead to a faulty transcript. Also, through the use of restriction

enzyme analysis using methyl-sensitive isoschizomers, such as Msp I and Hpa II, it would be possible to determine the level of DNA methylation which could ultimately lead to decreased transcription. Transcription of the gene could be studied through Northern blot analysis.

The introduction of both acidic and basic chitinases into carrot makes available host material with which to study the influence of these enzymes on fungal pathogen development. Acidic chitinases are believed to act during the early stages of pathogen attack, i.e. direct action on hyphae invading intercellular spaces, while basic chitinases are believed to function at a later stage when cell breakage releases the vacuolar contents (Broglie *et al.* 1991; Mauch and Staehelin 1989). Chitinases may possibly also have an indirect role by releasing defense-related elicitors from fungal cell walls which trigger the host defense reactions (Keefe *et al.* 1990). Chitinases engineered into higher plants have been shown to provide enhanced protection against fungi (Benhamou *et al.* 1993; Broglie *et al.* 1991; Dunsmuir *et al.* 1992) or to have no effect (Neuhaus *et al.* 1991a; Nielsen *et al.* 1993). Recently, Benhamou *et al.* (1993) reported that transgenic canola plants constitutively expressing a basic bean endochitinase gene affected invading hyphae of *Rhizoctonia solani* by breaking down chitin, disrupting the cells and causing protoplasm leakage. The influence of chitinases in the transgenic carrot plants described here in response to fungal infection is currently being studied.

Appendix I
# Construction of plasmid pGA492-CHN

Materials and Methods

Preparation of Binary Vector

Promoter expression vector pGA492, a 12.0 kb low copy plasmid containing a chimeric gene (nopaline synthase promoter fused to the NPT II coding region and nopaline synthase 3' end), a tetracycline-resistance gene, and a polylinker with multiple cloning sites (An, 1987) (Fig. 9) was obtained from Dr. A. Kermode (Simon Fraser University, British Columbia). The vector was multiplied by transforming *E. coli* DH5α cells (BRL) as per manufacturer's instructions. Small scale purification of plasmid DNA (miniprep) was carried out with the Magic Minipreps DNA Purification System™ (Promega, Cat. # A7100). The purified vector was linearized using restriction enzymes Cla I and Eco RI (BRL) as per supplier's recommendations.

After restriction enzyme digest, the linearized vector was electrophoresed on a 0.6% (w/v) low melting point agarose gel. Gel fragments containing the linearized vector were excised using a scapel, under low intensity UV light. Excised gel fragments were placed in a microfuge tube and placed at 70 °C until the agarose melted. The DNA fragments were purified and precipitated using the Magic PCR Preps DNA Purification System for Rapid Purification of DNA Fragments™ kit (Promega, Cat.# A7170).

Figure 9. Schematic diagram of the binary expression vector pGA492. The vector contains a chimeric nopaline synthase promoter fused to the NPT II gene, a tetracycline-resistance gene, and a polylinker with multiple cloning sites (An 1987).
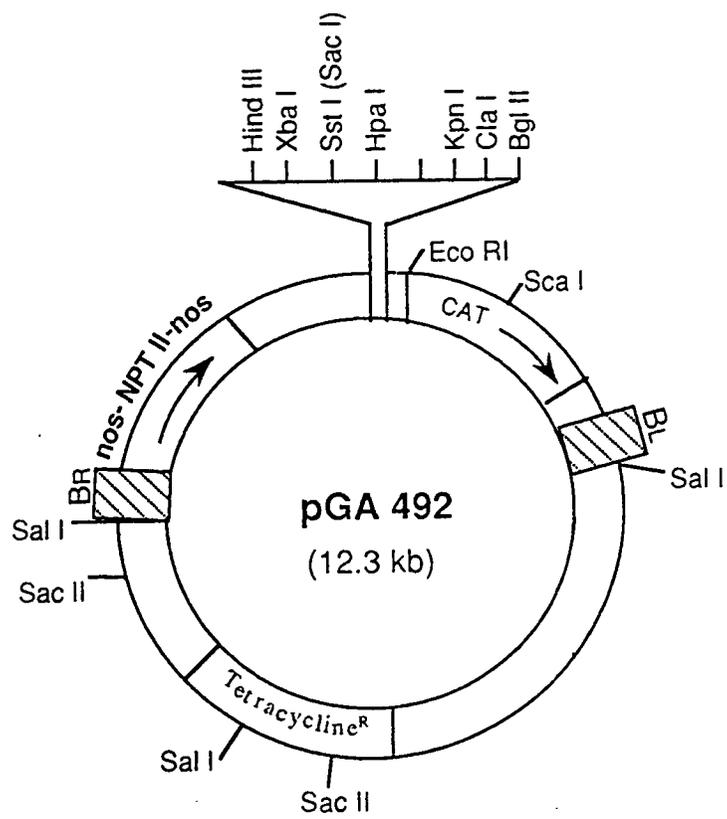
Preparation of Chitinase Insert

Vector pK35CHN-641 was supplied in *E.coli* HB101 cells by K. Broglie (E.I du Pont de Nemours, Delaware, U.S.A). pK35CHN0641 is comprised of the CaMV 35S promoter (965 bp) fused to a 1.5 kb fragment of the bean chitinase 5B coding region and nos 3'end. This is contained in a pBR322-based plasmid which also contains a 3.5 kb Eco RI fragment bearing the neomycin phosphotransferase (NPT) I gene of Tn903 and a chimeric gene consisting of the nopaline synthase (NOS) promoter joined to the *Tn5* NPTII coding sequence followed by the 3' end of octopine synthase (OCS) (Fig. 10). The plasmid DNA was isolated from HB101 cells in the same manner as pGA492, followed by restriction digest with Cla I and Eco RI. The digested products were electrophoresed on a 0.7% agarose gel, and the 2.5 kb fragment consisting of the CaMV 35S promoter-chitinase-nos 3' was purified from the gel (as above).

Construction of pGA492-CHN

For the construction of this plasmid, 1 μg of chitinase insert DNA and 0.2 μg of linearized vector pGA492 DNA (total volume =10 μL) were dissolved in 6 μL of ligation reaction mix (2.5X ligation buffer, 25mM dithiothreitol,250 mg mL^{-1} BSA, and 2.5 mM ATP), and incubated at 13 °C with 1.0 μL of T4 ligase. After 2 hours the sample was added 100 μL of dilute ligation reaction mix (108 μL + 114 μL dH$_2$O) and incubated overnight at 4 °C.

Confirmation of Vector Construction

The resulting ligated vector (Figure 2) was reproduced by transforming DH5α cells (BRL). The tetracycline-resistant transformants were analysed for the presence of the new construct by purifying the plasmid with the Magic

Figure 10. Structure of the intermediate plasmid pK35CHN641 provided by E.I
du Pont de Nemours, Delaware, U.S.A. The plasmid contains an Eco RI-Xho II
DNA fragment encoding the chimeric 35S-chitinase gene from bean (Broglie *et
al.* 1991).
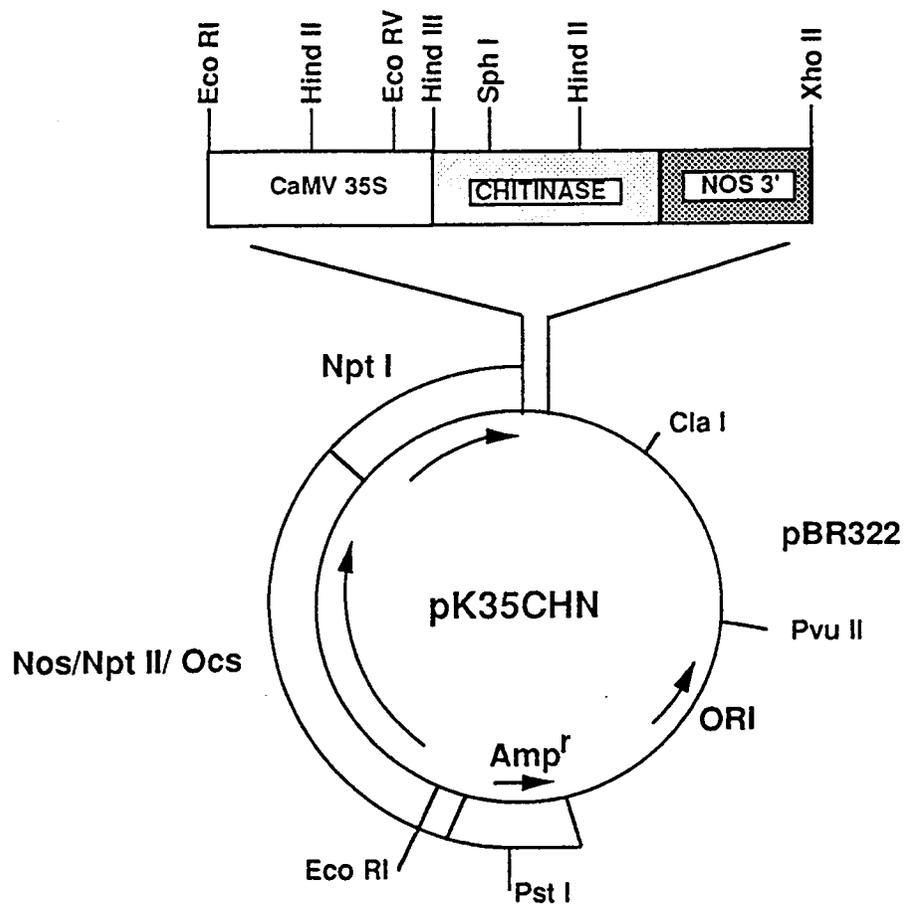
Minipreps™ DNA Purification System, and cleaving the DNA with ClaI and EcoRI to yield the desired 12.0 kb and 2.5 kb fragments.

Triparental Mating

The triparental mating procedure involves the conjugation of three bacteria, *E. coli* DH5α containing pGA492-CHN (kanamycin-resistant), *E. coli* HB101 containing the mobilization plasmid pRK2013 (kanamycin-resistant), and *A. tumefaciens* strains MOG101 (nopaline), MOG301 (octopine) and EHA105 (leucinopine). Each of the *Agrobacterium* strains carry a rifampicin selectable marker. In the triparental mating procedure, the pRK2013 plasmid mobilizes into *E. coli* DH5α containing pGA492-CHN plasmid. Once within the *E. coli* cell, pRK2013 provides transfer proteins which act on the pGA492-CHN plasmid mobilizing it into *A. tumefaciens*.

The details of the mating procedure were as follows. Overnight cultures of the *E.coli* and *Agrobacterium* were started. Both *E.coli* strains were grown in 10 mL of LB plus 100 mg mL^{-1} kanamycin at 37 °C, while *A. tumefaciens* was grown in 10 mL LB (no antibiotics) at 29 °C. The cells were diluted back and grown to log phase. Fifty μL from each of the cultures was spotted onto a piece of millipore paper overlaying an LB plate. The plate was incubated at 29 °C overnight. A loopful of the grown bacteria was streaked on selective Minimal Medium (Turk *et al.* 1991) containing 100 mg mL^{-1} kanamycin, 50 mg mL^{-1} rifampicin, and 250 mg mL^{-1} spectinomycin (spectinomycin when selecting for *A. tumefaciens* MOG101 and MOG301). The plates were incubated at 29 °C for 2-3 days. Bacterial colonies which successfully grew on the respective selective media were cultured in LB medium containing 100 mg mL^{-1} of kanamycin.

Confirmation of pGA492-CHN presence in <u>Agrobacterium</u> strains

Presence of the construct was confirmed by purifying the plasmid with the Magic Minipreps™ DNA Purification System, and digesting with Cla I and EcoR I to yield the desired 12.0 kb and 2.5 kb fragments.

Results

All bacterial plasmid minipreps yielded DNA which could be used directly for restriction digestion without further manipulation. Electrophoresis of the restriction digests using Cla I and Eco RI of the plasmids isolated from *Agrobacterium* strains showed a 12.0 kb and a 2.5 kb band corresponding to the linearized pGA492 vector and the chitinase insert respectively (Fig. 11).

Figure 11. Restriction enzyme digest of constructed vector pGA492-CHN using Cla I and Eco RI. DNA fragments were electrophosed, stained with ethidium bromide and viewed under UV light.

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