INVESTIGATIONS ON GENETIC TRANSFORMATION OF TOBACCO AND CANOLA WITH POTENTIAL ANTIFUNGAL GENES

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

in the Department of Applied Microbiology and Food Science

University of Saskatchewan

Saskatoon

By

Adrienne Eugenie Woytowich
January, 1997

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfillment

of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

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Spring 1997

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Investigations on Genetic Transformation of Tobacco and Canola with Potential Antifungal Genes

In nature there are several forms of plant-microbe interactions. Some of these interactions are beneficial to the plants while others are harmful. Most plants have the ability to distinguish between microbial pathogenic and symbiotic associations. In pathogenic relationships some plants synthesize antimicrobial products such as hydrolytic enzymes and phytoalexins whereas in symbiotics there is a coexistence of plant and microbes. Symbiotic nodule formation is a result of nitrogen-fixing bacteroids being encapsulated in healthy leghaemoglobin-containing tissues, whereas, pathogen attack induces the activation of a number of defense mechanisms. It is the activation of these defense mechanisms which leads to physical and biochemical changes within the plant. In attempts to create tobacco and canola plants that show an increase in disease resistance transformations were undertaken with the *chiB* gene of *Serratia liquefaciens* and a synthetic *amp2* gene which codes for a potential antimicrobial gene. However, after several transformation attempts with DNA vectors carrying *chiB* it was revealed that neither *chiB* containing tobacco nor canola plants could be regenerated using the *Agrobacterium*-mediated transformation procedure. In every transformation undertaken it was found that the infected discs and cotyledons did not swell normally, turned yellow and failed to form calli. The oncogenicity of the wild type *Agrobacterium tumefaciens* strain C58 was also tested in the presence of the *chiB* construct on various plants and it was found that this strain failed to form tumors around the infected wound sites. However, tumors did form around the wound sites that were inoculated with the same strain which carried only the DNA vector. The lack of calli and gall formation was confirmed to be due to the expression of *chiB* as inhibition of its expression by the introduction of mutations led to infected tobacco discs swelling normally and forming calli within four weeks of infection. As well, the replacement of the *chiB*-containing plasmid with

a broad host range plasmid led to the renewal of the oncogenic ability of the strain. Thus, it is being proposed that *Agrobacterium*-mediated transformations require a signal, similar to the Nod factors of *Rhizobium,* for calli and tumor formation to occur, but that the *chiB* gene product somehow inactivates this signal.

An antimicrobial gene, *amp2,* was synthesized based on the protein sequence of a protein isolated from the seeds of *Mirabilis jalapa.* The resulting gene product was found to display strong antimicrobial activity *in vitro.* The coding sequence of *amp2* was transferred to the genomes of tobacco and canola and protein extracts isolated from *amp2*-containing T_o tobacco and canola transgenic plants were found to lack antimicrobial activity. Assays undertaken with proteins extracted from T_1 and T_2 homozygous and heterozygous plants revealed that low levels of antimicrobial activity could be observed only from the progeny of the tobacco line AW708-1 when using extremely high concentrations of protein that had been desalted. However, none of the transgenic canola and tobacco seedlings tested showed any increase in tolerance towards the pathogen *Rhizoctonia solani.*

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ACKNOWLEDGMENTS

I would like to extend my sincere thanks to my supervisor, Dr. G. Selvaraj of the Plant Biotechnology Institute (PBI), National Research Council of Canada, for his continuous advice, guidance and support throughout this study. I also acknowledge my co-supervisor, Dr. G.G. Khachatourians of the Department of Applied Microbiology and Food Science, University of Saskatchewan, for his expert guidance and the confidence that he instilled in me throughout this study. I would like to thank Dr. W. Steck and Dr. K. K. Kartha, past and current Director Generals of PBI, for allowing me to undertake my research at the National Research Council. During the course of this study I received assistance from Mr. D. Schwab who synthesized the required oligonucleotides and Mr. B. Panchuk who carried out the DNA dideoxy sequencing and I thank them for their help. I extend special thanks to Mrs. Rozina Hirji for helping me learn some of the required laboratory techniques and being a good friend at all times. A special thank you is also extended to fellow graduate student Ravi Jain for his interesting conversations and suggestions. Finally, I thank the other members of my committee, Professors V. Misra, R. Morrall and W. M. Ingledew, for their helpful suggestions throughout this study. The financial support of the University of Saskatchewan and the College of Agriculture is acknowledged.

Finally, I thank my husband, Robert, and my parents, Dr. Peter and Mrs. Eugenie Nikiforuk, for their love, support and encouragement throughout my years at university. You taught me that I can accomplish anything as long as I put mind and heart to it. A very special acknowledgment goes to my daughter Maureen, who arrived in the middle of this study. It was nice to know that no matter how long or rough my day might have been you were always there, waiting for me with a smile on your face. Thank you for being so patient and understanding when mommy had to go to the laboratory to do an experiment instead of playing with you. Maureen, this thesis is dedicated to you.

ABSTRACT

Most plants have the ability to distinguish between pathogenic and symbiotic associations and to respond accordingly although there are similarities between pathogenic and symbiotic modes of infections. Symbiotic nodule formation is a result of nitrogen-fixing bacteroids being encapsulated in healthy leghaemoglobin-containing tissues, whereas attack by a pathogen induces the activation of a number of defense mechanisms. It is the activation of these defense mechanisms which leads to physical and biochemical changes within the plant. Originally, the main objective of this thesis was to determine if it is possible to produce transgenic tobacco and canola plants which showed increased resistance to plant pathogens by introducing the *chiB* gene of *Serratia liquefaciens* and a synthetic gene (*amp2*) coding for an antimicrobial peptide derived from *Mirabilis jalapa,* alone and in combination. However, experiments revealed that the presence of the *chiB* gene suppressed the oncogenic ability of *A. tumefaciens*, therefore preventing regeneration of transgenic plants. As well, transgenic tobacco and canola plants carrying *amp2* did not show increased resistance to plant pathogens.

The *chiB* open reading frame (ORF), under the control of the 35S-35S promoter module, was used to transform tobacco and canola plants. However, after several transformation attempts it was found that *chiB*-containing tobacco or canola plants could not be regenerated using the *Agrobacterium*-mediated transformation

procedure. It was found that in every transformation the discs and cotyledons did not swell normally, turned yellow and failed to form calli. The oncogenicity of the wild type *Agrobacterium tumefaciens* strain C58 was tested in the presence of the *chiB* construct on various plants and it was found that this strain failed to form tumors around the infected wound sites. However, tumors did form around the wound sites that were inoculated with the same strain which carried only the vector. The lack of callus and gall formation was confirmed to be due to the expression of *chiB*. Inhibition of its expression by the introduction of mutations led to infected discs swelling normally and forming calli within four weeks of infection. As well, the replacement of the *chiB*-containing plasmid with a broad host range plasmid led to the renewal of the oncogenic ability of the strain. From these results and other experiments it is being proposed that *Agrobacterium*-mediated transformations require a signal, similar to the Nod factors of *Rhizobium*, for callus and tumor formation to occur, but that the gene product of *chiB* somehow inactivates this signal.

The antimicrobial gene *amp2* was synthesized and the resulting gene product was found to display strong antimicrobial activity *in vitro*. The coding sequence of *amp2* was transferred to the genomes of tobacco and canola by *Agrobacterium*-mediated genetic transformation and protein extracts isolated from *amp2* - containing T_0 tobacco and canola transgenic plants were found to lack antimicrobial activity. Assays undertaken with proteins extracted from T_1 and T_2 homozygous and heterozygous plants revealed that low levels of antimicrobial activity could only be observed from the progeny of the tobacco line AW708-1 when using extremely high concentrations of desalted plant protein extracts (3 mg/50 μL). However, none of the transgenic canola and tobacco

seedlings tested showed any increase in tolerance towards the pathogen *Rhizoctonia solani.*

ORGANIZATION OF THE THESIS

The organization of this thesis deviates from the traditional format used in most theses published in the biological sciences at the University of Saskatchewan to allow for a more logical presentation of the results. The original objective of this study was to determine if it is possible to create fungal pathogen resistant transgenic tobacco and canola plants by introducing a series of antimicrobial genes, alone and in combination, into their genomes. While transgenic lines carrying an anitimicrobial peptide gene could be obtained (see section 4.0 entitled "Genetic Manipulation of a Synthetic Antimicrobial Peptide Gene in Tobacco and Canola"), it was observed that transgenic lines carrying a particular bacterial chitinase gene (*chiB*) could not be obtained by *Agrobacterium*-mediated genetic transformation of tobacco or canola. Considering that gene transfer by *A. tumefaciens* is an intensively investigated area of research and one that is routinely practiced in generating transgenic lines of tobacco and canola, it was a puzzling observation. This observation was investigated further and a systematic study showed that a functional *chiB* gene in *A. tumefaciens* prevented genetic transformation of plants. These experiments raised several interesting questions regarding the biology and biochemistry of *Agrobacterium* plant interactions and, as such, these results warranted presentation as a coherent section. Accordingly, this thesis contains two chapters, each with its own literature review. Considering that most of the materials and methods are

common to both chapters, one "Material and Methods" section is provided to avoid redundancy.

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LIST OF ABBREVIATIONS

Amp	ampicillin
*arg*F	ornithine carbamoyltransferase
BA	6-benzyl-aminopurine
bp	base pairs
β-ME	β-mercaptoethanol
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
cfu	colony forming units
cpm	counts per minute
CTAB	hexadecyltrimethyl ammonium bromide
ddH_2O	double distilled water
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanidine triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
dut	deoxyuridine triphosphatase
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
*end*A	DNA-specific endonuclease I
g	gram
Gm	gentamycin
*gyr*A	DNA gyrase, subunit A
h	hour

*hsd*R	host restriction (endonuclease R)
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kDa	kiloDaltons
Km	kanamycin
*lac*Z	β-D-galactosidase gene
min	minute
m^+_k	modification positive (*E. coli* K-type)
mRNA	messenger RNA
NAA	1-naphthylacetic acid
nptII	neomycin phosphotransferase gene
nt	nucleotide
OL	oligodeoxyribonucleotide
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	Polyethylene glycol
pfu	*Pyrococcus furiosus*
φ80*lac*Z	M15, partial deletion of *lac*Z
PIPES	piperazine-N-N'-bis[2-ethane sulfonic acid]
PMSF	phenymethylsulphonyl fluoride
PolIk	Klenow (large) fragment of the *E.coli* polymerase I
PVP	polyvinylpyrrolidone
r^-_k	restriction endonuclease negative (*E. coli* K-type)
*rec*A	general recombination, DNA repair and induction of phage
*rel*A	regulation of RNA synthesis, stringent factor
SDM	site-directed mutagenesis
s.e.	standard error
Sp	spectomycin
SSC	sodium saline citrate
ssDNA	single-stranded DNA

*sup*E	suppressor of amber mutations
TCA	trichloroacetic acid
T_o	primary transgenic plants
T_1	F_1 progeny of primary transgenic plants
T-DNA	transfer DNA
*thi*1	thiamine thiazole requirement
Ti	tumor inducing plasmid
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
2,4-D	2,4-dichlorophenoxyacetic acid
u	units
ung	uracil-DNA-glycosylase
v	volume
V	volts
w	weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-gluc	glucuronidase

1.0 INTRODUCTION

In nature there are several forms of plant-microbe interactions. Some of these interactions are beneficial to the plants while others are harmful. For example, there are a number of diseases of canola, such as blackleg and stem rot, that are due specifically to the deleterious association of certain fungi with the host plant.

When plants are challenged by potential pests and pathogens a complex progression of molecular and biological interactions occurs including a number of defense responses (Collinge and Slusarenko, 1987). Some of the major defense responses elicited by plants include the accumulation of antimicrobial phytoalexins (Davis *et al.*, 1986), enhancement of wall-bound hydroxyproline-rich glycoproteins (Graham and Graham, 1991), deposition of phenolic compounds and lignin (Barber *et al.*, 1989) and the elaboration of several classes of pathogenesis-related proteins with antifungal and/or antibacterial properties (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988; Broekaret *et al.*, 1989).

Chitinases catalyze hydrolysis of the β-1,4 linkages of the N-acetyl-D-glucosamine polymer of chitin to oligomers and monomers (Cabib, 1988). Chitinases are produced by a wide variety of organisms such as bacteria (Clarke and Tracey, 1956), insects (Koga *et al.*, 1983), fungi (Bartnicki-Garcia, 1968) and all higher plants (Abeles *et al.*, 1970; Boller *et al.*, 1983; Graham and Sticklen, 1994). In all of these species, except

for bacteria and higher plants, the substrate chitin is also present. It has been proposed, therefore, that chitinase play a role in the differentiation, development and growth of the former.

In healthy plants chitinase levels are either very low or undetectable, but upon infection they increase significantly. For this reason, and because chitin is a major cell wall component of most fungi, it has been proposed that chitinase is a pathogenesis-related protein that acts against chitin-containing pathogens (Abeles *et al.*, 1970; Boller, 1985). Several studies have demonstrated that chitinase levels increase in response to endogenously produced ethylene, pathogen attack and fungal elicitors (Pegg and Young, 1982; Chappell *et al.*, 1984), and also that chitinases may play multiple roles in enhancing a plant's defense against invading pathogens. Since chitinase has been shown to act synergistically in the lysis of fungal hyphal tips (Mauch *et al.*, 1988), it is thought that it may degrade the pathogen's cell wall or disrupt its deposition thus leading to death. As well, it is thought that chitinase may cause the release of pathogen cell wall fragments which may then act as elicitors of active host defense responses.

Plants also produce other proteins which play important roles in the control of microorganisms. Many of these proteins are small disulfide-linked peptides of approximately 50-100 amino acids in length that display antifungal properties, but their modes of action are not fully known. Such peptides have been isolated from the seeds of maize (Duvick *et al.*, 1992), *Mirabilis jalapa* (Cammue *et al.*, 1992), radish (Terras *et al.*, 1995) and *Amaranthus caudatus* (Broekaert *et al.*, 1992) and all have been found to be toxic to numerous plant-pathogenic fungi and Gram-positive bacteria.

Since a number of research reports clearly indicate that introduction of bacterial or plant chitinase genes into tobacco and canola enhance their resistance to fungal pathogens, it is of interest to determine if this resistance can be further enhanced by the presence of other antimicrobial genes. Accordingly, the original objective of this study was to determine if a synthetic antimicrobial peptide gene (*amp2*), based on the protein sequence of the antimicrobial peptide 2 of *M. jalapa,* and the *chiB* gene of *Serratia liquefaciens* alone or in combination increases the resistance of tobacco and *Brassica napus* to certain plant pathogens. During the course of this study it was found that it was not possible to transform tobacco or *B. napus* with *Agrobacterium tumefaciens* Ti vector constructs containing *chiB*, or *chiB* and *amp2*. This discovery prompted an investigation of why the *chiB* gene of *S. liquefaciens* inhibits the transformation process. This thesis, therefore, in addition to describing the experiments on transgenic plants carrying a synthetic *amp2* gene, describes a series of experiments designed to establish why *chiB* inhibits the transformation process in tobacco and *B. napus* and why tumorigenesis is inhibited in several different dicots by the presence of the *chiB* gene of *S. liquefaciens*.

2.0 MATERIALS AND METHODS

2.1 Bacterial strains and cultivation procedures

The *Escherichia coli, A. tumefaciens* and *Bacillus megaterium* strains used in this project are listed in Table 2.1. The growth media are listed in Table 2.2. The cultures used were started from frozen laboratory stocks. The *E. coli* strains were grown by inoculating 5 mL of 2YT medium, with an appropriate antibiotic if required, and incubating at 37°C for 18-24 h with aeration. The *A. tumefaciens* strains and *B. megaterium* were grown in 2YT and TYS media, respectively, and incubated at 28°C. To prepare the strains for long-term storage, 0.8 mL of the culture was mixed with 0.8 mL of 40% (v/v) sterile glycerol and added to presterilized cryovials (Fisher Scientific Co., Edmonton) and immediately frozen in liquid nitrogen. One batch was placed at -70°C and the other at -90°C as master stock. Working stocks were prepared by streaking frozen icicles onto agar plates which were stored at 4°C for up to one month.

Table 2.1 Bacterial strains used in this study

Strain	Relevant Characteristics		Source or Reference
	Chromosomal Markers	Plasmid	
Escherichia coli			
DH5α	*end*A1 *hsd*R17 *thi*1 ($r^-_k m^+_k$) *sup*E44 *rec*A1 *gyr*A95 *rel*A1 (*arg*F-*lac*ZYA) U169Δ80 M15λ$^-$	nil	Bethesda Research Laboratory
CJ236	*dut*1 *ung*1 *thi*1 *rel*A1	pCJ105; Cm^r; $F^/$	Kunkel *et al.*, (1987)
Agrobacterium tumefaciens			
GV3101		pMP90; Gm^r	Koncz and Schell, (1986)
C58		pGV3850, Tc^r	Van Haute *et al.*, (1983)
281		pTiBo542, Rif^r	Hood *et al.*, (1986)
348		pTiA6, Rif^r	Hooykaas and Mozo, (1994)
Bacillus megaterium ATCC 13632			American Type Culture Collection

*arg*F, ornithine carbamoyltransferase (arginine biosynthesis); *dut*, deoxyuridinetriphosphatatase; *end*A, DNA-specific endonuclease I; Gm^r, gene for gentamycin resistance; *gyr*A, DNA gyrase, subunit A; *hsd*R, host restriction (endonuclease R);φ 80*lac*Z M15, partial deletion of *lac*Z; *lac*Z, β D-galactosidase; m^+_k, modification positive (*E. coli* K-type); r^-_k, restriction endonuclease negative (*E. coli* K-type); *rec*A, general recombination, DNA repair and induction of phage 1; *rel*A, regulation of RNA synthesis, stringent factor; *sup*E, suppressor of amber mutations; *thi*1, thiamine thiazole requirement; *ung*, uracil-DNA-glycosylase

Table 2.2 Composition of growth media

Growth Media	Composition (weight/liter of solution)	Reference
TYS	Bacto-tryptone, 100 g; yeast extract, 50 g; NaCl, 50 g; and 1 M NaOH, 20 mL	
2YT	Bacto-tryptone, 160 g; yeast extract, 100 g; NaCl, 50 g and 1 M NaOH, 20 mL	Miller, 1972
MS	The following macro and micronutrients are in g/L NH_4NO_3, 1.6; KNO_3, 1.9; $CaCl_2 \cdot 2H_2O$, 0.44; $MgSO_4 \cdot 7H_2O$,0.37; KH_2PO_4, 0.17; KI, 0.0008 H_3BO_3,0.0062; $MnSO_4 \cdot 4H_2O$, 0.0223;$ZnSO_4 \cdot 7H_2O$, 0.0086; $CuSO_4 \cdot 5H_2O$, 0.000025; $CoCl_2 \cdot 6H_2O$, 0.000025; sequestrene 300 Fe (EDTA-ferric salt), 0.043 and 3% sucrose. pH 5.6 $\pm$ 0.1	Murashige and Skoog, 1962
B5 vitamins	All vitamins are in g/L; inositol, 0.1; nicotinic acid, pyridoxine·HCl, 0.001 and thiamine·HCl, 0.01	Gamborg *et al.*, 1968
Co-cultivation (tobacco)	MS, B5 vitamins, 2 mg/L 2,4-D (2,4-dichloro-phenoxyaceticacid). 2,4-D was prepared by first dissolving in a very small amount of ethanol and then making up the volume with water.	
Shooting (tobacco)	MS, B5 vitamins, 2.5 mg/L BAP (6-benzyl-aminopurine), 0.1 mg/L NAA (1-naphthylacetic acid), 100 mg/L kanamycin sulphate and 500 mg/L carbenicillin. BAP and NAA were prepared by initially dissolving in a small volume of 0.5 N HCl (with heating) and ethanol, respectively, and then making up the volume with water.	
Rooting (tobacco)	MS, B5 vitamins, 0.1 mg/L NAA, 100 mg/L kanamycin sulphate and 500 mg/L carbenicillin.	

Continued .....

Table 2.2 Composition of growth media continued

Co-cultivation (*B. napus*)	Murashige Minimal organics with 3% sucrose, pH 5.8
Medium 2 (*B. napus*)	Murashige Minimal organics with 3% sucrose, 4.5 mg/L BA, pH 5.8, 500 mg/L carbenicillin.
Medium 3 (*B. napus*)	Murashige Minimal organics with 3% sucrose, 4.5 mg/L BA, pH 5.8, 500 mg/L carbenicillin, 20 mg/L kanamycin.
Medium 4 (*B. napus*)	Murashige Minimal organics with 3% sucrose, 1 mg/L BA, pH 5.8, 500 mg/L carbenicillin.
Medium 5 (*B. napus*)	MS with Gamborg's vitamins, 3% sucrose, 0.1 mg/L NAA, 20 mg/L kanamycin.

2.2 Growth media, buffers and solutions

Double deionized water was used for the preparation of buffers and solutions. All of the chemicals used were of reagent grade. The compositions of the media are presented in Table 2.2. For solid media used for bacterial cultures Bacto agar (Difco Laboratories, Detroit) was added at a concentration of 1.5% (w/v). The solutions, media and buffers were sterilized by autoclaving for 20 min at 121°C (15 lb/in^2).

For tobacco tissue culture, Murashige and Skoog's basal medium (Murashige and Skoog, 1962) with Gamborg's vitamins (Sigma, Mississauga) was used and for *Brassica* tissue culture Murashige Minimal organics medium was used. For solid plant tissue culture growth medium phytagar was used at a concentration of 0.7% (w/v).

The compositions of the various buffers and solutions used in this study are described throughout the Materials and Methods section. For the antibiotics and those solutions that are heat labile filter sterilization was employed using a pre-sterilized 0.22 μm filter unit (Millipore) . The pH of the buffers was determined using a Fisher Accumet Model 825 MP pH meter. If it was necessary to adjust the pH, NaOH, HCl or acetic acid at 10 mM was used.

Antibiotics when used were as indicated. Ampicillin, kanamycin, gentamycin and spectinomycin were used at concentrations of 25 and 50 μg/mL for liquid and solid media, respectively. Carbenicillin was prepared freshly, when required, and was used at a concentration of 500 mg/L.

To identify recombinant plasmids in *lac* α complementation experiments the chromogenic substrate of β-galactosidase, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, GIBCO/BRL, Burlington) was used at a concentration of 0.01% (w/v). The

X-gal solution was prepared in N,N-dimethyl formamide (BDH Chemicals, Saskatoon, Canada) and used immediately. IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma, Mississauga) was prepared in water and was included at a concentration of 0.5 mM as a gratuitous inducer of the *lac* promoter.

Yeast extract, tryptone and agar were purchased from BDH Chemicals Canada Ltd. (Saskatoon). The MS with Gamborg's vitamins, various inorganic salts, kanamycin and growth regulators were obtained from Sigma Chemical Co. (Mississauga). The phytagar and Murashige minimal organics were obtained from GIBCO BRL (Burlington) and the carbenicillin from Ayerst (Montreal).

2.3 DNA Procedures

2.3.1 Estimation of DNA concentrations

The concentration of the DNA which was used in the various experiments was determined using a spectrophotometric method. This method involved using a Beckman DU-6 spectrophotometer to determine the absorption of a sample of DNA (500 μL total volume) at 260 nm and 280 nm. An absorbance unit of 1.0 at 260 nm was taken to represent approximately 50 μg/mL of double stranded DNA (Maniatis *et al.*, 1982). By comparing the measured absorbance at 280 nm and 260 nm the purity of the DNA samples was determined. A ratio of approximately 1.8 between the 260 nm and 280 nm values indicated the DNA was pure, while values below 1.8 indicated that the sample was contaminated with proteins or phenol. This comparision was only used until research on the validity of nucleic acid purities monitored by the 260/280 absorbance ratios indicated that an R value of 1.8 corresponded to a mixture of approximately 60% protein and 40%

nucleic acids (Glasel, 1995). Once it became apparent that the widely used R260/280 method for determining the purity of nucleic acid preparations was a very poor choice, this ratio was no longer used. When precision measurement was not essential, the concentration of DNA was estimated visually by comparison of known amounts of DNA standards with the ethidium-bromide induced fluorescence of the sample DNA. The DNA standards used were λDNA digested with *Hind* III and φX174 DNA digested with *Hae* III.

2.3.2. Large scale plasmid DNA preparations and purification

Large scale plasmid preparations were made using a Qiagen® plasmid maxi kit. The term Qiagen® is a trademark of Qiagen® Inc. (Chatsworth, California). All of the centrifugations used in the large scale plasmid DNA preparation and purification procedure were carried out at 4°C unless otherwise noted. In this procedure a 100 mL 2YT culture of the strain carrying the plasmid to be isolated was grown at 37°C for 18 h under antibiotic selection for the plasmid. The cells were collected by centrifuging at 5500 x g for 10 min and resuspended in 4 mL of P1 buffer (100 μg/mL Rnase A, 50 mM Tris·HCl and 10 mM disodium ethylenediamine tetraacetic acid, pH 8.0). Once the cells were resuspended, 20 mg of lysozyme was added to the suspension. The suspension was then mixed and incubated for 10 min at room temperature. In order to complete lysis of the cells, 4 mL of alkaline SDS solution (0.2 M NaOH and 1% sodium dodecyl sulphate) was added to the suspension which was incubated at room temperature for 5 min. The chromosomal DNA and proteins were precipitated by adding 4 mL of 3 M potassium acetate buffer (294 g/L potassium acetate and 115 mL/L acetic acid, pH 5.4) and chilling

on ice for 10 min. The supernatant containing the plasmid DNA was isolated by centrifugation at 12,500 x g for 30 min. A Qiagen®-tip 100 column was equilibrated by applying 4 mL of QBT buffer (750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100) and allowing the column to empty by gravity flow. The supernatant containing the plasmid DNA was loaded onto the equilibrated column and allowed to enter the resin by gravity flow. Once the supernatant had passed through the column, the column was washed twice with 10 mL of QC buffer (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 8.5). The DNA was eluted from the column by adding 5 mL of QF buffer (1.25 M NaCl, 50 mM Tris·HCl, 15% ethanol, pH 8.5). The plasmid DNA was then precipitated with the addition of 0.7 volumes of isopropanol and subsequent centrifugation at 15,000 x g for 30 min. The resulting pellet was washed with 70% ethanol and dried at room temperature for 10 min. The dried pellet was resuspended in 100 µL of TE buffer (10 mM Tris·HCl and 1 mM disodium EDTA, pH 8.0).

2.3.3. Small scale preparation of plasmid DNA

Rapid plasmid DNA extractions were required to screen a large number of clones routinely. Small scale preparations of plasmid DNA were prepared using the SDS alkaline method (Maniatis *et al.*, 1982). Bacterial cultures were grown overnight at 37°C in 2-5mL of 2YT with an appropriate antibiotic to which the plasmid in question confers resistance. The cells were harvested by centrifuging at 1000 x g for 5 min in a bench top centrifuge (Damon/IEC Division) and resuspended in 100 µL of GTE buffer (50 mM glucose, 25 mM Tris·HCl and 10 mM EDTA, pH 8.0) by vortexing. The cell suspension was incubated at room temperature for 10 min and transferred to a microfuge tube. Two

hundred microliters of freshly prepared alkaline/SDS buffer (1% SDS and 0.2 M NaOH) was added to the cell suspension. This mixture was mixed gently and then placed on ice for 10 min prior to the addition of 150 μL of 5 M potassium acetate buffer (pH 5.4). The sample was chilled on ice for another 10 min and the protein and chromosomal DNA were removed from the plasmid DNA by centrifuging twice for 10 min in an Eppendorf microcentrifuge (Brinkmann, Model 5414). The resulting supernatant was transferrred to a fresh tube and 2.0 volume of 95% ethanol was added so as to precipitate the plasmid DNA. After further incubation at room temperature for 10 min, the plasmid DNA was recovered by centrifuging the suspension in an Eppendorf microcentrifuge for 10 min. The plasmid DNA was washed with 70 % ethanol to remove any precipitated salts and dried at 40° C for 30 min in a vacuum desiccator. The dried pellet was resuspended in 40 μL of TE buffer and for subsequent restriction enzyme digest 10 μL of each sample of DNA was used. In cases where it was necessary to further purify the DNA a phenol:chloroform extraction was performed. The DNA was extracted once with an equal volume of phenol:chloroform (1:1) and then with an equal volume of chloroform:isoamyl alcohol (24:1). Each extraction was carried out in an Eppendorf Mixer for 5 min and the phases were separated by centrifuging in a microfuge for 5 min.

2.3.4 Isolation of bacterial genomic DNA by the miniprep method

In this protocol bacteria grown in a liquid culture were lysed and proteins were removed by digestion with proteinase K. Cell wall debris and polysaccharides were removed by selective precipitation with CTAB. The high molecular weight DNA was then recovered by isopropanol precipitation. A 5 mL 2YT culture was inoculated with the

bacterial strain of interest. The culture was grown for 48 h at 30°C, transferred to Eppendorf tubes and centrifuged in a microcentrifuge for 2 min. The supernatant was discarded and the pellet resuspended in 567 μL TE buffer by repeated pipetting. Once the cells were resuspended 30 μL of 10% SDS and 3 μL of 20 mg/mL proteinase K were added to each tube to give a final concentration of 100 μg/mL proteinase K in 0.5% SDS. The tubes were mixed thoroughly and incubated for 1 h at 37°C. Once the incubation was complete 100 μL of 5 M NaCl was added and the contents of each tube was mixed thoroughly. This was a very important step as CTAB-nucleic acid precipitate will form if the salt concentration drops below 0.5 M at room temperature. Once the salt concentration was above 0.5 M, 80 μL of the CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) was added to each tube and incubated for 10 min at 65°C. To remove the CTAB-protein/polysaccharide complexes an equal volume of chloroform/isoamyl alcohol was added and after mixing the tubes were centrifuged in a microcentrifuge for 5 min. The aqueous, viscous supernatant was removed to fresh microcentrifuge tubes and an equal volume of phenol/chloroform/isoamyl alcohol added. After centrifuging for 5 min the supernatant was transferred to fresh tubes containing 0.6 volume isopropanol. The contents of the tubes were mixed gently and centrifuged for 1 min to collect the DNA. The DNA was washed with 70% ethanol and allowed to air dry. Each pellet was then resuspended in 100 μL TE buffer.

2.3.5 Isolation of uracil containing single stranded plasmid DNA

To introduce new restriction sites into plasmid DNA by site directed mutagenesis (SDM) it was necessary to isolate single stranded DNA (ssDNA) from the

desired plasmid. The recombinant plasmids were mobilized into the Dut$^-$Ung$^-$ *E. coli* strain CJ236 which has lower levels of dUTP pyrophosphatase and uracil *N*-glycosylase. The lower level of dUTP pyrophosphatase allows for an elevated concentration of dUTP which then competes with dTTP for incorporation into the DNA. The uracil *N*-glycosylase enzyme removes the incorporated dU from the DNA, but when it is absent or less abundant uracil-containing DNA is not repaired. The uracil containing ssDNA is then packaged into phage particles by infecting CJ236 with the helper phage M13K07. The uracil containing ssDNA can then be used for SDM.

The desired plasmids were transferred to the CJ236 strain and their integrity checked. Single colonies carrying the plasmid were grown overnight in 5 mL 2YT medium with 30 μL/mL chloramphenicol (to select for the F$'$ plasmid) and the antibiotic required for selection of the plasmid. Fresh 20 mL 2YT cultures were inoculated with 1 mL of the overnight culture and allowed to grow for 1 h. At this time 20 μL of uridine (250 μg/mL) was added to the 20 mL culture which was then allowed to grow for 5 min. At this point, the cells were infected with 200 μL of the helper phage M13K07 (10^{10} plaque forming units/mL). The culture was then shaken vigorously at 300 rpm in a gyratory shaker (New Brunswick Scientific Co.) for 1 h. After 1 h, kanamycin (70 μg/mL) was added to the culture to select for infected cells and the culture was then grown overnight.

The cells were pelleted by centrifuging at 9000 x g for 15 min and the supernatant was collected and transferred to a 30 mL Corex tube. The phage particles were precipitated by adding 4 mL of a 25% PEG 8000/3 M NaCl solution, followed by gentle mixing and incubation on ice for 30 min. The precipitate was collected by

centrifuging for 30 min at 10,500 x g in a Beckman swinging bucket rotor. The pellet was allowed to air dry before being resuspended in 1 mL of TE. The ssDNA was extracted by phenol:chloroform and chloroform:isoamyl alcohol and precipitated from the aqueous phase by adding 0.1 volume 3 M sodium acetate (pH 5.2) and 2 volumes of cold ethanol. The sample was then incubated at -70°C for 30 min.

2.3.6 Site-directed mutagenesis

Site-directed mutagenesis (Zoller and Smith, 1984; Kunkel *et al.*, 1987) was performed with minor modifications. To separate the plasmid uracil containing ssDNA from the chromosomal DNA and the helper phage DNA present in the sample, the uracil containing ssDNA was eluted from a 1% (w/v) agarose gel prior to setting up the site-directed mutagenesis reaction. In this process, the uracil containing ssDNA is used as a template for the synthesis of a complementary strand containing the desired mutated DNA sequence. The resulting heteroduplex is transferred to a suitable *E. coli* Ung^+ strain where the uracil *N*-glycoylase enzyme releases uracil from the uracilated template strand, while the newly synthesized strand with thymidine is left intact. This release leads to the production of apyrimidinic sites in the template strand which inhibits DNA synthesis and susceptibility of the template strand to apyrimidinic endonuclease. Most of the progeny that arise are from the complementary strand and, therefore, carry the desired mutation.

The primer required for the reaction, which had been phosphorylated at the 5' end by a polynucleotide kinase reaction, was annealed to the ssDNA (3 pmoles of primer/pmole ssDNA) in the presence of 1 μL of 10 x buffer A (0.2 M Tris·HCl pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl and 0.01 M DTT), by incubating for 5 min at 55°C. The

reaction was allowed to cool at room temperature for 10 min after which 1.5 μL of 10 x buffer B (0.25 M Tris/HCl pH 7.5, 0.1 M $MgCl_2$ and 0.01 M DTT), 2 μL of a stock solution of dATP, dCTP, dTTP and dGTP (2 mM), 1 μL of 20 mM ATP, 4 units of T4 DNA ligase, and 10 units of Pol IK were added to the annealed DNA. The reaction was incubated at room temperature for 18 h after which it was used to transform DH5αF′ competent cells. The DNA was isolated from the transformants by the rapid plasmid preparation protocol previously described and screened with the appropriate restriction enzymes for the desired changes. All of the nucleotide changes introduced by SDM were also confirmed by sequencing the regions where the changes had been introduced.

2.3.7 Synthetic oligonucleotides

All oligonucleotides used in this study were synthesized and purified by Mr. D. Schwab of the Plant Biotechnology Institute, National Research Council, Saskatoon, according to the method of Gait (1984). The oligonucleotides were created by a phosphoramidate synthesis procedure using a Biosearch 8750 DNA synthesizer (New Brunswick Scientific Co.), purified by polyacrylamide gel electrophoresis, and visualized on a fluorescent TLC plate using UV light. Alternatively, an HPLC-based procedure was used to purify the oligonucleotide.

2.3.8 Isolation of plant DNA

Total genomic DNA was extracted from all the tobacco and plants by the CTAB method developed by Doyle and Doyle (1990). To ensure all of the soil and microorganisms were removed from the leaves used for the extraction of DNA, the leaves

were first washed several times with distilled water, placed in 70% ethanol for 30 sec and then rinsed once again with distilled water.

In this procedure, 5 mL of CTAB isolation buffer (2% hexadecyltrimethyl ammonium bromide, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA and 100 mM Tris·HCl, pH 8.0) was preheated in sterile centrifuge tubes in a 60°C water bath. One gram of fresh leaf tissue was frozen with liquid nitrogen and pulverized into a fine powder with a mortar and pestle. The frozen ground tissue was scraped directly into the preheated buffer. The suspension was gently mixed and incubated at 60°C for 30 min with occasional swirling. The solution was extracted once with chloroform-isoamyl alcohol (24:1;v:v) and mixed gently. After extraction the suspension was centrifuged (1600 x g) for approximately 10 min at room temperature. The aqueous phase was removed with a pipet and transferred to a clean tube to which 2/3 volume of cold isopropan-2-ol was added. The mixture was gently mixed to precipitate the DNA. At this stage the DNA precipitate was visible and was recovered by centrifuging the tube at 500 x g for 2 min. The supernatant was carefully removed and the resulting pellet incubated for 20 min in 1 mL of wash buffer (76% ethanol, 10 mM CH_3COONH_3). The DNA was collected by centrifuging for 10 min at 1600 x g. The pellet of DNA was allowed to air dry and was then resuspended in 500 μL of TE buffer.

The carbohydrates were removed from the DNA preparation by precipitating the DNA by adding 200 μL of 5 M NaCl and 1 mL of cold ethanol. The DNA was pelleted by centrifugation and washed with 70% ethanol, dried and redissolved in 200 μL TE buffer.

2.3.9 Electrophoresis of DNA in agarose gels

Agarose gel electrophoresis was used for both preparative and analytical purposes. The fragments of DNA were separated by electrophoresis through either a 1% or 4% agarose gel, depending on the size of fragment, in 1 x TAE buffer (4.8 g/L Tris·HCl, 1.1 mL acetic acid, 2 mL 5 M disodium EDTA, pH 8.0). The agarose gels were prepared by autoclaving the appropriate amount of agarose in 100 mL of 1 x TAE so that the agarose would dissolve. The solutions were allowed to cool to 60°C and then poured into gel trays. In order that the DNA fragments could be visualized under UV light ethidium bromide was added to the cooled agarose at a concentration of 1 μg/mL. The gels were submerged with a sufficient amount of TAE buffer prior to loading of the DNA samples. The DNA samples to be loaded were mixed with loading buffer which consisted of 10 X TAE, 0.2% bromophenol blue, 1% SDS, 25% Ficol and 0.75 units/mL of Rnase B. Depending on the size of the fragments and the specific type of electrophoresis equipment used (Pharmacia GNA 200 and IBI Model QSH), the gels were run for 1 h at 100 volts or 18 h at 20 volts.

The size of each DNA fragment was estimated by comparing it to standards of known size which were run in adjacent lanes. The standards used on all the gels were bacteriophage lambda DNA digested with *Hind* III and φ X 174 DNA digested with *Hae* III. These provided size standards of 23.13 kb, 9.41 kb, 6.55 kb, 4.36 kb, 2.32 kb, 2.03 kb, 1.37 kb, 1.07 kb, 0.87 kb, 0.63 kb, 0.310 kb, 0.281 kb, 0.234 kb, 0.194 kb and 0.118 kb. The size of each fragment was determined relative to a semi-log plot of fragment size against the distance traveled by the DNA standards. After electrophoresis, the gels were

visualized on a short wave UV light trans-illuminator and photographed with a Polaroid MP-4 camera and Polaroid 57 instant film.

2.3.10 Isolation and elution of DNA from an agarose gel

The DNA that was required for the probe preparation and subcloning procedures was isolated by the means of agarose gel electrophoresis. The DNA was visualized and the desired fragments were excised using the "Preparative" mode of the Foto/Prep-1 (Fotodyne) illuminator and a clean razor blade. The "Preparative" mode was used because it employs a longer wave UV and reduces the intensity of the light source so that UV-mediated degradation of the DNA does not occur.

The DNA was eluted from the segment of agarose using the Qiaex DNA gel extraction protocol developed by Qiagen® Inc. (Chatsworth). At the present time the components of all of the Qiagen® buffers included in the kit used have not been published or revealed by the company. The gel slice, containing the DNA fragment, was placed in an 1.5 mL Eppendorf tube and solubilized by adding 300 μL of QX1 buffer per 100 mg gel and 10 μL of QIAEX per 5 μg of DNA. The sample was incubated at 50°C for 10 min. To ensure that maximum binding occurred the sample was vortexed every 2 min during this incubation. In the cases where the DNA fragment was larger than 10-kb the sample was mixed gently by flicking and inverting the tube rather than using a vortex. The sample was then centrifuged for 30 s in a microcentrifuge. The supernatant was carefully removed with a pipet and the resulting pellet was resuspended in 500 μL of QX2 buffer. This step was repeated twice and the resulting pellet was then washed twice with 500 μL of QX3 in the same manner just described. After the final wash the pellet

was allowed to air dry. The DNA was eluted from the QIAEX by the addition of 20 μL TE buffer. The pellet was gently resuspended in the TE buffer and then incubated at room temperature for 10 min. To elute the DNA fragments larger than 10-kb the elution temperature was increased to 50°C. The sample was then centrifuged for 30 s and the resulting supernatant, which contained the DNA, was transferred to a clean tube.

2.3.11 Enzymatic modifications of DNA

2.3.11.1 Restriction endonuclease digestion

All of the restriction endonuclease digests that were used in various experiments were carried out under the conditions recommended by the supplier of the specific enzyme (BRL Life Technology, Burlington or New England BioLabs, Mississauga). Restriction enzyme digests were done to analyze recombinant clones, to digest plant genomic DNA and to isolate specific DNA fragments for subcloning and modification. Typically the digests were done in a 20 μL reaction volume using 1 unit of the enzyme for 1 μg of DNA, where a unit of enzyme is defined as the amount of enzyme required to completely digest 1 μg of DNA at the recommended temperature in 1 h.

In those cases where two restriction enzymes had to be used they were mixed together when the reaction conditions were optimum for both of the enzymes. When the conditions were not optimum, the reactions were performed sequentially with an intermediate step to remove the first enzyme and buffer.

For Southern blot analysis, 10-20 μg of plant genomic DNA was digested in a 50 μL volume using 20-50 u of restriction enzyme for 6 h. An aliquot of the reaction was

then removed and checked to ensure digestion was complete. If it was not complete the reaction was incubated overnight.

2.3.11.2 Formation of DNA with blunt ends

To allow ligation between the DNA fragments which had been digested with restriction enzymes that created heterologous ends in some of the cloning experiments the cohesive ends were rendered blunt. This procedure involved inactivating the restriction enzymes by a single phenol/chloroform extraction followed by an ethanol precipitation step. The cohesive ends were filled in a reaction using 1 mM deoxyribonucleotide triphosphates (from a stock containing 2 mM each of dCTP, dATP, dTTP and dGTP), 2 units of the Klenow fragment DNA polymerase I (Pol IK) and 0.1 x volume of Klenow buffer (10 mM Tris·HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT) followed by incubation at room temperature for 30 min. The reaction was then stopped by placing the reaction tube at 68°C for 15 min. The DNA was purified by extracting the proteins with a phenol:chloroform mixture. The DNA was ethanol precipitated, washed with 70% ethanol and resuspended in water or TE buffer.

2.3.11.3 Dephosphorylation

To prevent self-ligation of vector DNA, which leads to a reduction in the frequency of recombinant plasmids, the 5' phosphate group was removed from restriction-digested vector DNA by treatment with 3 units of calf intestinal phosphatase (Promega, Madison) in a 20 µL reaction volume. A ten-fold concentrated reaction buffer (0.5 M Tris·HCl pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$ and 10 mM spermidine) was

supplied by Promega (Madison). The mixture was incubated for 1 h at 37°C after which the reaction was terminated by heat-inactivation at 65°C for 15 min. The reaction mixture was extracted first with a phenol:chloroform (1:1) mixture and then with a chloroform:isoamyl alcohol (1:1) mixture. The DNA was ethanol-precipitated, washed with 70% ethanol and resuspended in an appropriate volume of TE buffer.

2.3.11.4 DNA ligation

Ligation reactions were performed to covalently link DNA fragments with blunt or compatible cohesive ends. The reactions were carried out in a 20 μL volume and consisted of 2 units of T4 DNA ligase, 0.1 x volume ligase buffer (50 mM Tris·HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 26 μM NAD^+, 25 μg/mL BSA) and the DNA fragments in a 2:1 insert-to-vector molar ratio. After incubating overnight at 14°C, the aliquots were used directly for the transformation of competent cells.

2.3.12 Dideoxy DNA sequencing

All of the nucleotide sequencing undertaken in this project was done using a modification of the dideoxy method developed by Sanger *et al.* (1977). The DNA to be sequenced was prepared as described in section 2.3.2. Nucleotide sequencing was done by Mr. B. Panchuk of the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, in an Applied Biosystems Model 370A sequenator using oligonucleotide primers synthesized at the Institute.

2.3.13 Southern transfer

DNA extracted from tobacco and *B. napus* plants (10-20 μg) was digested with the desired restriction enzyme by incubating with 20 to 50 u of the enzyme for 4 h at 37°C. The digested samples were run on a 1% TAE agarose gel containing ethidium bromide (1 μg/mL) at 20 V for 8 h. The presence of discrete repetitive DNA bands and fragments less than 1 kb were taken as indicators of complete digestion of the high molecular weight DNA. A picture of the gel was taken with a UV-fluorescent ruler which was used as a reference for marking the distance traveled by the DNA standards. The DNA was denatured by soaking the gel in a denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 h. The gel was neutralized by soaking it in a neutralizing buffer (1 M Tris·HCl pH 8.0, 1.5 M NaCl) for an additional hour and the DNA then transferred from the gel to a nylon membrane (Micron Separation Inc.) by the Southern transfer method (Southern, 1975; Maniatis *et al.*, 1982). This method involves placing the gel, with the bottom side facing upwards, onto a platform covered with a sheet of 3M Whatman paper. The platform separates two reservoirs containing 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate pH 7.0) and this allows the buffer to flow through the filter paper and into the gel. The nylon membrane was soaked for 2 min in 6 X SSC and then placed on top of the gel carefully ensuring that no air bubbles were present. A 7 cm stack of absorbent paper towels was placed on top of the membrane and covered with a 500 g glass plate. This set up allows transfer of the DNA from the gel to the membrane by capillary action. This transfer was allowed to proceed overnight at which time the membrane was soaked for 10 min in 6 X SSC and baked for 2 h at 80°C in a vacuum oven to affix the transferred DNA

onto the membrane. The membrane was either used immediately or stored at 4°C in a sealable plastic bag until needed.

2.3.14 Radioactive DNA probes

Radioactive probes were created using a Random Primer Kit (BRL Life Technologies Inc., Burlington). All of the required reaction components, except for the radiolabelled deoxy 5'-triphosphate ([α ^{32}P]-dCTP), were supplied with the Random Primer Kit.

The DNA to be labelled was purified as previously described in section 2.3.10. Twenty-five ng of the DNA (in 23 μL of water) was placed into a 1.5 mL screw-capped vial (Sarstedt) and incubated at 95°C for 5 min so that denaturation would occur. After 5 min the tube was immediately placed on ice at which time the following were added to the reaction mixture: 2 μL each of dATP, dGTP and dTTP, 15 μL Random Primer buffer mixture, 5 μL [α-^{32}P]dCTP (10 μCi/μL) and 1 μL of Klenow (3U). The mixture was centrifuged briefly and incubated at room temperature for 2 h. The reaction was stopped by the addition of 2 μL of 0.2 M EDTA, pH 7.5. Unincorporated nucleotides were removed from the labelled DNA by using a nick column (Pharmacia). After passing through a nick column the probe was collected as a 400-μL sample to which 50 μL of salmon sperm DNA was added. One μL of the labelled probe was placed into a scintillation counter vial containing 10 mL of scintillation fluid and checked for the incorporated radioactivity using a 1217 Rackbeta liquid scintillation counter (LKB

Wallac). For the most part, radioactive probes with a specific activity of $1x10^8$ counts per min (cpm) per μg of DNA were routinely obtained.

2.3.15 DNA-DNA hybridization

The membranes to be probed were soaked in water and prehybridized in 50 mM piperazine-N-N'-bis [2-ethane sulfonic acid] (PIPES) pH 6.5, 100 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, 5% SDS and 2% blocking reagent (Boehringer Mannheim, Dorval) for 1 h at 65 °C (Virca *et al.*, 1990). The radiolabelled probe was mixed with 200 μg of salmon sperm DNA and 1 mL of QuikHyb ™ solution (Strategene, La Jolla). The solution was then incubated at 100°C for 5 min in order for the probe to denature. The denatured probe was added to the hybridization solution and the membrane hybridized at 65°C for 24 h. Both prehybridization and hybridization were carried out in roller bottles in a Hybaid mini-hybridization oven (Bio/Can Scientific). The membranes were washed in the same rollers at 65°C, 55°C or 45°C, depending on the experiment, as follows: 45 min with buffer A (40 mM NaH_2PO_4 pH 7.2, 1 mM EDTA, 5% SDS and 0.5% blocking reagent) and 30 min with buffer B (40 mM NaH_2PO_4 pH 7.2, 1 mM EDTA and 1% SDS). The signal was then monitored by a hand-held Thyac Geiger counter (Victoreen).

2.3.16 Polymerase chain reaction (PCR)

When it was necessary to amplify DNA fragments or introduce new restriction sites the polymerase chain reaction (PCR) (Friedman *et al.*, 1990) was employed. Polymerase chain reactions were performed in a Hybraid Bio/Can Scientific Thermal Reactor. The reactions were run using 10 pg of template DNA, 20 pmole of the

oligonucleotides, 1 x PCR buffer (10X: 500 mM KCl, 100 mM Tris·HCl pH 8.3, 15 mM $MgCl_2$ and 0.1% gelatin), 8 μL of a 10 mM stock of dNTPs and 1 μL of Taq (BRL) or *pfu* (New England BioLabs) polymerase in a total reaction volume of 100 μL. Prior to the addition of the dNTPs and the enzyme, the reaction mixture was incubated in the thermocycler at 95°C for 5 min to completely denature the DNA. The reaction mixture was then maintained at a temperature of 80°C at which time the dNTPs and enzyme were added. The fragment was then amplified by incubation at 95°C for 1 min, at 45°C for 1 min, and at 72°C for 1 min, for a total of 25 cycles. Upon the completion of 25 cycles the reaction was extended by incubating at 72°C for 15 min. Amplified DNA to be used for further manipulations, such as cloning, was purified using a Qiagen PCR purification kit.

2.3.17 PCR product purification

PCR products required for restriction digests and subsequent cloning were purified using the QIAquick PCR purification kit produced by Qiagen Inc. (Chatsworth). For every 100 μL PCR reaction 500 μL of buffer PB was added and the solution mixed well. A QIAquick spin column was placed into a 2 mL collection tube and the sample was loaded into it. The column was centrifuged in an Eppendorf microfuge for 60 sec. The column was washed with 0.75 mL of buffer PE and centrifuged for another 60 sec. The PE buffer was drained from the tube and the column was centrifuged for 30 sec to remove any residual buffer. The column was placed in a fresh 1.5 mL Eppendorf tube. To elute the DNA, 50 μL of TE buffer was added to the column which was then centrifuged for 30 sec.

2.4 Protein procedures

2.4.1 Extraction of proteins from plant material

Crude extracts of proteins were extracted from the leaves of tobacco and canola plants. To ensure that soil and microorganisms were not present on the leaves they were first washed several times in distilled water and briefly washed with 70% ethanol. The leaves were then blotted and weighed. Five grams of leaves were placed in a previously chilled mortar and pestle with 1.25 g of polyvinylpyrrolidone (PVP), which helped to remove many of the phenolics that were present in the leaves. Liquid nitrogen was added to the mortar and the leaves were ground to a very fine powder. Five hundred microliters of β-mercaptoethanol and 2.5 mL of grinding buffer (0.01 M Tris pH 7.5, 10 mM $MgCl_2 \cdot 6H_20$, 1 mM PMSF and 5 mM DTT) were added to the frozen material and it was allowed to thaw until it formed a thick paste. The paste was transferred to Eppendorf tubes and centrifuged for 15 min. The supernatant was transferred to a fresh tube and centrifuged for another 15 min. The concentration of the crude extract of proteins was determined by using a BioRad (Mississauga) kit based on the method of Bradford (1976). The samples were used immediately or stored at -70°C for up to one week. Those samples that needed to be desalted were passed through a Sephadex G-25 column (PD-10 columns; Pharmacia, Dorval) equilibrated with the same buffer. The desalted proteins were eluted in 3.5 mL of the extraction buffer.

2.4.2 Extraction of bacterial protein

Proteins were extracted from the various bacterial strains by the use of a French Pressure Cell (Aminco). The bacterial strains were grown in 5 mL of 2YT media which

contained, if required, the appropriate antibiotic for selection or inducer for 18 h at 37°C. The bacterial cells were pelleted by centrifugation at 1,000 x g for 10 min and resuspended in 1 mL of grinding buffer (0.01 M Tris·HCl pH7.5, 10 mM $MgCl_2 \cdot 6\ H_2O$, 1 mM PMSF and 5 mM DTT). The resulting suspensions were passed through a prechilled French Pressure Cell at a pressure of 12,000 psi. The protein samples were used immediately or stored at -70°C for up to 2 days. Those samples requiring desalting were processed as described in section 3.4.1.

2.4.3 Estimation of protein concentration

The concentration of proteins isolated from plants and bacterial cultures was estimated by the method developed by Bradford (1976) using the reagents supplied by Bio-Rad and BSA as a standard. In this procedure protein samples were mixed in distilled water to a total volume of 800 µL. Two hundred microliters of the BioRad Protein Assay Reagent was then added to give a final volume of 1 mL. The samples were then incubated at room temperature for 10 min, after which the absorbance of each sample was measured at 595 nm in a Beckman DU-6 spectrophotometer.

2.4.4 Polyacrylamide gel electrophoresis (PAGE)

To resolve proteins on the basis of molecular mass SDS-PAGE was employed. The procedure used for SDS-PAGE was that of Laemmli (1970) as updated by Davis *et al.* (1986). The gels were cast and then run in a Mighty Small II (Hoeffer Scientific, Model SE 250) gel apparatus.

For the *amp2* project a 18% separating gel was prepared by mixing 18% (w/v) acrylamide/bis-acrylamide (29:1) and 0.375 M Tris·HCl pH 8.7. The solution was degassed for 5 min and the 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.0005% (v/v) TEMED were added. The gel was poured immediately and allowed to solidify. The stacking gel was prepared by mixing 3% (w/v) acrylamide/bis-acrylamide (29:1) and 0.125 M Tris.HCl pH 6.8. Following degassing 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.0005% (v/v) TEMED were added and the stacking gel was immediately poured onto the separating gel. Once the gel had solidified the apparatus was filled with SDS-PAGE running buffer (0.025 M Tris·HCl, 14.4% (w/v) glycine, 0.1 (w/v) SDS, pH 8.3).

2.4.5 Chitinase assay

Chitinase activity was measured in plants and bacterial strains by a procedure developed by Molano *et al.* (1977) which involved using tritiated-chitin (^{3}H-chitin). This chitinase assay is based on the insolubility of the reaction product in water. If chitinase is present in the sample being tested the ^{3}H-chitin will be degraded into the water-soluble oligosaccharides which can be separated from the insoluble ^{3}H-chitin by centrifugation and their radioactivity determined.

The procedure employed in this assay involved isolating crude protein extracts from plants or bacterial cultures as previously described. The reaction mixture contained, in a total volume of 200 µL, 0.05 M potassium phosphate, pH 6.3, ^{3}H-chitin ($1x10^6$ cpm) and aliquots of the crude protein extracts (25 µg). Two controls were used in each experiment and they consisted of 20 mM sodium acetate, instead of the crude protein

extracts, and boiled crude protein extracts. All of the reaction mixtures were incubated for 18 h at 37°C. Following the incubation period, 300 µL of 10% (v/v) trichloroacetic acid (TCA) was added to each tube. The suspensions were centrifuged in an Eppendorf centrifuge for 5 min in order to pellet the insoluble ^{3}H-chitin. Using a micropipet, 200 µL of the supernatant was removed from each reaction mixture and transferred to a scintillation vial which contained 10 mL of scintillation fluid. The radioactivity of each sample was then determined.

2.5 Transfer of plasmid DNA to bacteria

2.5.1 Transfer of plasmid DNA to *Escherichia coli* by the $CaCl_2$ method

E. coli cells were gentically transformed employing the procedure described by Morrison (1979). A fresh 25 mL 2YT culture medium, without antibiotics, was inoculated with 1 mL of an overnight culture of the desired strain. The strain was allowed to grow to an A_{550}= 0.5. The culture was then transferred to a chilled centrifuge tube and centrifuged at 5000 x g for 10 min at 4°C. All of the subsequent steps of the procedure were carried out on ice and all the buffers used were prechilled to 4°C. The cell pellet was resuspended very gently in 20 mL of ice cold 0.1 M $MgCl_2 \cdot 6H_2O$ and centrifuged once again for 10 min at 5000 x g. The cells were resuspended in 20 mL of ice cold 0.1 M $CaCl_2 \cdot 2H_2O$ and placed on ice for 30 min following which they were centrifuged for 10 min at 5000 x g. The competent cells were resuspended in 1 mL of $CaCl_2$-glycerol mix (86% 0.1 M $CaCl_2 \cdot 2H_2O$ and 14% glycerol) and 200 µL were transferred in to screw-capped vials. The cells were frozen in liquid N_2 and stored at -70°C until required.

Prior to transforming, the frozen competent cells were allowed to thaw on ice for 2 min. Once thawed, the cells were mixed with DNA (50-200 ng) and incubated for 30 min on ice. In order to facilitate DNA uptake the cells were heat shocked at 42°C for 2 min. After adding 1 mL of 2YT, the cells were incubated at 37°C for 1 h to allow for expression of the antibiotic resistance gene. The cells were centrifuged for 30 sec in a microfuge and resuspended in 200 μL of TYS and plated on to a selection medium containing the desired antibiotic. Transformed cells were purified by streaking for single colonies on 2YT plates containing the appropriate antibiotic.

2.5.2 Transfer of plasmid DNA to *Agrobacterium tumefaciens* by the freeze-thaw method

The freeze-thaw method of Holsters *et al.* (1978) was employed to introduce the binary plasmids carrying the various genes into *A. tumefaciens*. This method was employed instead of tri-parental mating (An *et al.*, 1988) so as to avoid the possibility of plasmid rearrangement which sometimes occurs during mating. The competent cells used in this procedure were prepared by inoculating 50 mL of 2YT, containing the appropriate antibiotics, with 2 mL of an overnight culture and incubating at 28°C to an A_{600}= 0.5-1.0. The cells were pelleted by centrifuging for 5 min at 5000 x g and then resuspended in 1 mL of ice-cold 20 mM $CaCl_2$. The competent cells that were not being used immediately were stored at -70°C in 100 μL aliquots. Approximately 1 μg of plasmid DNA was added to the frozen cells which were then allowed to thaw at 37°C for 5 min. One mL of 2YT medium was added to the cells which were then incubated for 4 h at 28°C. The cells were centrifuged for 30 sec in a microfuge and resuspended in 100 μL of 2YT medium.

The cells were plated onto 2YT plates containing the required antibiotics to select *Agrobacterium* cells carrying both the Ti and binary plasmid. The plates were incubated at 28°C for 2-3 days until the colonies appeared.

2.6 Plant tissue culture methods

2.6.1 Genetic transformation of *Brassica napus* by *A. tumefaciens*

The method used to transform *B. napus* is based on that of Moloney *et al.* (1989) with a few modifications as recommended by Mr. J. Hammerlindl and Dr. W.A. Keller of the Plant Biotechnology Institute, National Research Council, Saskatoon.

Seed sterilization : *B. napus* seeds were sterilized by soaking them in 95% ethanol for 15 sec and then in 50% Javex with one drop of wetting agent Tween-20 for 15 min. In a fumehood the Javex™ solution was then removed and replaced with 0.025% mercuric chloride plus one drop of Tween-20. The seeds were incubated in this solution for 10 min and then rinsed several times with sterile distilled water. Once dried the sterile seeds were aseptically plated on 15x60 mm Petri dishes containing 1/2 strength hormone-free MS and 1% sucrose. The petri dishes were placed in Magenta GA7 jars in order to allow germinating seeds to grow taller and straighter thus making it easier to harvest the cotyledonary explants for infection. The plates were then incubated at 25°C with 16h light/8h dark for 4 days.

Explant preparation: Sterile forceps were used to grasp both petioles of the two cotyledons just above the point where they join the hypocotyl. Using the lower edge of the forceps as a guide the petioles were cut off with a sharp sterile scalpel as close to the

apical meristem as possible without including it. The cotyledons were dropped onto a petri dish containing Medium 1 (Table 2.2) until they were ready to be infected.

Co-cultivation: The *A. tumefaciens* GV3101 strain, carrying the T-DNA plasmid to be transferred, was grown in 2YT media, containing the appropriate antibiotics, at 28°C for 2 days. The cells were harvested by centrifugation and washed two times with 5 mL of 2YT. The cells were next poured into a 15x60 mm petri dish. Using sterile forceps each explant was picked up and the cut end of the petiole dipped into the culture. The cut ends of the infected petioles were then embedded onto the surface of Medium 1. The plates were sealed with Parafilm and incubated at 25°C with 16 h light/8 h dark for three days.

After three days, the explants were transferred to plates containing Medium 2 (Table 2.2), sealed with Parafilm and incubated for seven days at 25°C with 16 h light/8 h dark. After this incubation the explants were transferred to plates containing Medium 3 (Table 2.2) in deep (25x100 mm) petri dishes. At this time if any of the explants had developed roots they were cut off and discarded. As well, any meristematic tissue that was inadvertently included with the explant and developed into shoots was removed and discarded to avoid high numbers of escapees. The plates were then sealed and incubated as previously described. The explants were transferred to fresh Medium 3 plates every two weeks. After three weeks on Medium 3 some shoots began to develop. Since the kanamycin selection tends to be leaky, many non-transformed shoots also developed. These shoots tended to be white or purple and were discarded. Shoots that originated from transformed cells tended to be dark green with a distorted club-like morphology and thick, fleshy leaves.

The regenerates were transferred to Medium 4 where they were allowed to grow into shoots with normal morphology. This usually occurred with a single transfer, but in some cases two or three transfers were required to obtain a normal shoot. Once good shoots had developed the callus growth present at the base of the stem was removed and the shoot was transferred to Medium 5 (Table 2.2) in tall jars for subsequent rooting. With the development of good root systems the plants were removed from the jars and most of the agar was removed by holding the roots under running water. The plants were transferred to moist potting soil and covered for five days to maintain high humidity. Once the plants had hardened they were uncovered and maintained in a growth room with 16 h light/ 8 h dark and a temperature of 22°C.

2.6.2 Genetic transformation of tobacco by *A. tumefaciens*

The transformation protocol used is based on the method originally described by Horsch *et al.* (1985). In this protocol healthy tobacco leaves, approximately 10-15 cm in length, were used from plants prior to bolting. The leaves were first washed in running water in order to remove any loose soil amd other particulate matter that might be present. All of the following steps were then carried out in a laminar flowhood. The leaves were first incubated in 70% ethanol for 2 min and then transferred to a bleach solution (8% Javex and 0.1% Tween-20) for 5 min. Following this, each leaf was rinsed four times with sterile double distilled water. One-third of the leaf near the petiole region was cut off and discarded. The remainder of each leaf was cut into squares approximately 1 cm x 1cm with a sterile scalpel and each square was transferred to a petri dish containing sterile water. While the pieces were in the sterile water, a 10 mL

overnight culture of *A. tumefaciens* was centrifuged for 10 min at 5000 x g. The supernatant was removed and the cells resuspended in 10 mL of fresh 2YT medium. The bacterial suspension was decanted into a petri dish and the leaf pieces were submerged in it and incubated with gentle shaking for 30 min. The leaf pieces were blotted dry on Whatman 3MM paper and transferred, with the underside down, to plates containing co-cultivation media (Table 2.2). Each leaf piece was pressed down to ensure that all the cut edges were in contact with the medium. The plates were sealed with Parafilm and incubated for three days at 25°C with 16 h light/ 8 h dark.

Controls were established for each transformation undertaken. Approximately 20 uninfected leaf discs were transferred to the co-cultivation media with kanamycin to test the kanamycin sensitivity of the leaves. The discs were also tested for their ability to form shoots by placing them on control media that lacked antibiotics.

After three days the pieces were transferred to selection medium. The edges of each piece were pressed down on to the media surface and this was repeated every four or five days as they tended to curl up. Shoots appeared on the leaf discs on the medium without antibiotics within two weeks. Untransformed leaf discs died within two weeks. Callusing on infected leaf discs occured within three weeks and shoot formation was visible within four weeks. Independent transformants were transferred onto fresh shooting medium and allowed to develop. Shoots were usually ready to be transferred to rooting media five weeks after infection. Six shoots per 10 cm x 2.5 cm Petri plate were transferred to rooting media and adequate roots normally formed within four weeks. The roots of the regenerated plants were gently washed with water to remove as much of the

agar as possible prior to being transferred to potting soil. The plants were covered for one week to conserve moisture until they were well established.

2.7 Growth and maintenance of the transgenic plants

Young transgenic tobacco and canola plants were transferred from rooting medium to 1 L covered pots and kept in the pots until they were well established. This took five weeks, after which the plants were transferred to 2 L pots to allow for further growth. The tobacco plants were grown in a walk-in growth chamber while the canola plants were kept in a small growth cabinet. All of the plants were grown under 16 h light/8 h dark with a day/night temperature of 22°C/20°C. The entire inflorescence of all the plants was covered with a fine-mesh cellophane bag (Super MicroBags; W.R. Grace and Company Canada Ltd., Cryovac Division) in order to prevent cross-pollination. Dry, mature siliques (canola) and capsules (tobacco) were collected and stored at room temperature after adding 10% captan to prevent the growth of molds.

2.8 Progeny analysis of transgenic tobacco plants

Segregation of kanamycin resistance in the T_0 and T_1 progeny of tobacco was followed by comparing the number of resistant and sensitive seedlings on 1/2 strength MS medium containing 100 μg/mL kanamycin sulphate. The seeds to be tested were sterilized in a 1.5 mL screw capped vial in 70% ethanol for 2 min, and in a bleach solution (8% Javex and 0.1% Tween-20) for 5 min. The seeds were then washed several times with sterile water and dried for 1 h in a Savant SpeedVac Concentrator with the cap of the tube ajar. The sterile seeds were sprinkled on the growth media and the plates were

incubated in 16 h of 700 μmol m^{-2} s^{-1} of light and day/night temperatures of 22°C/20°C for three weeks. Within two weeks the sensitive seedlings began to turn pale green and never grew beyond the cotyledonary leaf stage. The resistant seedlings, however, remained green and formed true leaves. The segregation ratios for kanamycin resistance in progeny seedlings were calculated and further evaluated statistically by Chi-square analysis.

2.9 GUS assays for identification of transgenic plants

Leaves, or portions of them, were removed from the plants and soaked in a GUS staining solution (1 mM X-gluc; 100 mM sodium phosphate; 10 mM EDTA; 0.1% Triton X-100) (Jefferson, 1987) for 24 h at 37°C. In order to remove the chlorophyll from the plant tissue, so as to determine if the tissure was positive for β-glucuronidase activity, the material was next soaked for 24 h in 100% ethanol. The ethanol was then removed from the samples and the tissue was placed under a steromicroscope for observation.

2.10 Antimicrobial assays

2.10.1 Plate assays for antibacterial activity

The antibacterial activity of the AMP2 protein was determined by plate assays using the test organism *B. megaterium*. A 5 mL culture of *B. megaterium* was grown to a late logarithmic phase in 2YT broth. A 2YT plate was overlaid with top agar containing 100 μL of the *B. megaterium* culture. While the top agar was hardening 25 μg of each of the protein extracts to be tested were placed onto sterile Whatman 3M discs (50 mm in diameter). Each disc was then placed on a plate and incubated overnight at 30°C.

Bacteriostatic or bacteriocidal activity was indicated by a clearance or halo around the disc.

2.10.2 Antifungal assays in soil

2.10.2.1 Preparation of inoculum for screening canola and tobacco

The inoculum used in this study was prepared by Mr. D.L. McKenzie of Agriculture Canada (Saskatoon). The cereal grain rye (*Secale cereale* L.) was chosen as the growth and carrier substrate due to its large ellipsoid shape. The inoculum was prepared by soaking 100 mL of grain in an equal volume of water in a 2 L polyethylene jar for 18 h. The jars were then autoclaved for 90 min at 121°C and 15 lb/in^2. When removed from the autoclave the jars were shaken to prevent seed packing. Once cooled, the jars were inoculated with plugs of actively growing *Rhizoctonia solani.* An isolate of *R. solani* AG2-1 was used for the canola assay while an isolate of *R. solani* AG1-1 was used for the tobacco assay. The grain cultures were incubated for two weeks at 25°C at which time all the grains were colonized. The inoculum was then removed from the jars and spread on tables for drying in circulating air at 25°C (Yang and Verma, 1992). Once the grain was dry, it was milled and sieved to a particle size of 0.6-1.2 mm diameter. The inoculum was then stored at 4°C until used.

2.10.2.2. Screening canola seeds in a growth chamber

For this study 1000 particles of the milled inoculum were thoroughly mixed with 1000 mL of air-dried, screened, soil-free growth medium (SFM) containing shredded peat moss, vermiculite, 0.5 cm maximum particle size washed sand and several macro- and

micro-nutrients (Stringham, 1971). Using a vacuum pick-up plate, 15 canola seeds were placed on a lightly packed surface of uninfested SFM in a plastic pot. The seeds were then covered with 100 mL of infested SFM containing approximately 100 viable particles of *R. solani* AG2-1 infested rye grain. As a control, non-transformed seeds of the same genotype were sown in the same manner. Four replicate pots were used for each plant tested. The pots were placed in a completely randomized design in a large metal tray in a growth chamber programmed for a 16 h day of 700 μmol m^{-2} s^{-1} of irradiance with day/night temperatures of 18°C/7°C, respectively. The pots were watered at regular intervals by flooding the tray for 2 h and then draining.

The criterion used to evaluate disease reactions was the mean percentage seedling emergence nine days after seeding. The *B. napus* plants that emerged were then categorized as either highly resistant, mildly resistant, susceptible or susceptible specifically to damping off (Yang and Verma, 1992).

2.10.2.3 Screening tobacco seedlings in a growth chamber

The fungal strain *R. solani* AG1-1 was used as a test organism to examine the antifungal tolerance of the transgenic tobacco plants. The inoculum was prepared as described in section 2.9.2.1 except that the grain was not milled. Tobacco seeds to be tested were placed in pots containing sterile vermiculite. Two weeks after the seeds had been planted 15 seedings in the cotyledon stage were transferred to trays that were 2/3 filled with sterile vermiculite. Two pieces of grain, containing the inoculum, were placed beside each seedling and the trays were then placed in a growth chamber with 16 h

light/8 h dark and a day/night temperature of 22°C/20°C. The seedlings were then examined every three days to determine their survival rate.

2.11 *Agrobacterium tumefaciens* infection of plants

To study the effect *A. tumefaciens* strains carrying different constructs have on tumor formation of various plant species a wound site was introduced onto the stem of the plant being studied using a sterile scalpel. The wound site was then inoculated with 50 µl of the desired bacterial culture with an OD of 1 at A_{595}. All of the experiments were done in duplicate and five different plant species were used. Each plant was inoculated with the *chiB*-containing strain and control strains which did not contain the vector or contained the vector, but lacked *chiB*.

3.0 PRESENCE OF A *SERRATIA LIQUEFACIENS chiB* CODING SEQUENCE IN *AGROBACTERIUM TUMEFACIENS* ABOLISHES GENETIC TRANSFORMATION OF PLANTS BY THE BACTERIA

3.1 Literature review

3.1.1 Biology of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a Gram-negative aerobic soil bacterium that is capable of saprophytic growth. This species of *Agrobacterium* is responsible for the crown gall disease of dicotyledonous plants. *Agrobacterium tumefaciens* infects a broad variety of plants through open wound sites and individual cells are infected at the site of infection which causes them to proliferate and form a gall (DeCleene and DeLey, 1976). The formation of a gall of disorganized callus tissue is caused by the transfer and integration of a fragment of bacterial DNA into the genome of the plant cells (Chilton *et al.*, 1977). The transferred DNA (T-DNA) directs the production of opines which are novel compounds that provide a source of nutrients to the colonizing bacteria at the site of infection. The T-DNA is present on the so-called tumor-inducing plasmid (Ti) which contains a much larger nontransferred segment (Chilton *et al.*, 1980). The latter includes genes which encode proteins that confer bacterial virulence and direct opine catabolism.

The T-DNA borders, two 25-bp direct repeats, define and delimit the T-DNA. It has been well documented that only the DNA between these borders is transferred and

that the borders are differentially utilized since transfer has been determined to be polar. The right border is critical for the transfer of DNA as the transfer must start from this end. Deleting or reversing the orientation of this border abolishes the T-DNA transfer (Peralta and Ream, 1985; Wang *et al.*, 1984). Manipulation of the left border, however, has little or no effect on the transfer of the DNA. It is interesting to also note that the border sequences are highly homologous which suggests that each may be capable of directing polar transfer. Of course, this could lead to the non-productive transfer of DNA away from the T-DNA. However, this does not occur because the sequence context of the T-DNA borders greatly influences their activity. Therefore, on the native Ti plasmid the sequences surrounding the left border attenuate polar transfer while the sequences surrounding the right border enhance it.

3.1.2 Virulence genes

A set of virulence genes (*vir*) are located on the Ti plasmid and they are very important as they code for functions that are involved in plant cell recognition, attachment, T-DNA excision, transfer and integration into the plant genome (Wang *et al.*, 1987; Stachel and Zambryski, 1986). Thus, the *vir* region of the Ti plasmid, which is approximately 30-kb and is organized into the eight complementation groups *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G, *vir*H and *vir*J, is responsible for providing most of the products that mediate T-DNA movement.

The products of *vir*A and *vir*G have been shown to recognize the signal molecules produced from wounded plant cells (Lee *et al.*, 1995). This recognition triggers the activation of other *vir* genes. The *vir*A product is an inner membrane protein that

recognizes and responds to the presence of plant phenolic compounds. VirA transduces this information to the VirG protein which acts as a transcriptional activator of its own gene and other *vir* genes. The VirC and D proteins are known to be involved in the generation and subsequent processing of the T-DNA copy. VirD specifically produces a site-specific endonuclease that cuts within the 25-bp right border of the T-DNA. This allows for the generation of a single-stranded T-strand that is subsequently transferred to the plant genome. The products of the *vir*B and *vir*E genes are involved in forming most of the structural components that facilitate T-DNA movement. Although *vir*H is not absolutely necessary for virulence, mutants have been found to have attenuated pathogenicity on certain host plants. It is possible that the *vir*H products allow the bacteria to survive in the presence of bacteriocidal or bacteriostatic plant compounds. Mutagenesis of the *vir* region on the Ti plasmid has revealed that *vir*J locus is induced by the plant-wound signal molecule, acetosyringone (Pan *et al.*, 1995). Examination of the amino acid sequence of *vir*J has revealed that it is similar to a region of the chromosomal gene *acv*B which is required for virulence. It is believed that *vir*J encodes the same factor required for tumorigenesis as *acv*B since it can complement the avirulent phenotype of an *acv*B mutant.

3.1.3 *Agrobacterium*-plant pathogenesis

3.1.3.1 Chemotaxis and attachment

Agrobacterium species are motile bacteria that possess a very sensitive chemotaxis system which responds to a wide range of sugars, amino acids and phenolic compounds such as acetosyringone. It is believed that chemotaxis plays a key role in the

early events of infection, and is often considered to be the first step in infection, as without cell-to-cell contact DNA transfer would rarely occur (Lippincott and Lippincott, 1969; Hawes *et al.*, 1988). Assays have been developed that measure translocation towards excised root tips and isolated root caps (Hawes *et al.*, 1988). Several mutants deficient in chemotaxis have been studied and when these mutants were assayed for their ability to form tumors on pea plants it was found that if they were used to inoculate the plants directly, they were fully virulent. However, when they were used to inoculate soil, which was dried and then used to grow plants, the nonchemotactic mutants were avirulent. If inoculated in sand instead of soil, they were virulent (Hawes and Smith, 1989). From these results Hawes and Smith (1989) concluded that chemotaxis is critical in certain soil types, but is most likely less important in less compact soils.

It has been suggested that the bacteria may be drawn to a large number of wound-released compounds, such as sugars and amino acids, due to the fact that well aerated soils tend to be poor in usable growth substances (Kennedy, 1987). Ashby and co-workers (1988) have found that the strain C58 is attracted to a group of phenolic compounds such as acetosyringone. Other groups studying the strain A348 failed to detect chemotaxis towards acetosyringone at any concentration (Hawes and Smith, 1989; Parke *et al.*, 1987). Strain A348, however, is attracted to other components of wound exudate such as some of the sugars.

The second step in infection and tumorigenicity is the binding of bacteria to plant cells. It is thought that specific receptors may be involved on both surfaces since the binding of *A. tumefaciens* is saturable and while some other genera of bacteria cannot compete for binding sites (Lippincott and Lippincott, 1969). Evidence also suggests that

attachment is a two-step process. In the first step *A. tumefaciens* binds to the plant cell surface as a single cell and in the second step it elaborates cellulose fibrils, in response to plant factors, that entrap the other bacteria. This then results in the formation of bacterial aggregates which also cause the bacteria to bind tightly to the plant cell surface (Matthysee *et al.*, 1981).

The chromosomal genes *chv*A, *chv*B, *exo*C and *att* of *A. tumefaciens* have been identified as playing a role in attachment to plant cells. Mutations in these genes strongly attenuate virulence as a decrease in the binding of the bacteria is observed (Douglas *et al.*, 1985). The *chv*A, *chv*B and *exo*C genes are required for the synthesis of a cyclic β-1,2-glucan which has been implicated in plant cell binding. ChvB is involved in the biosynthesis of the glucan and ChvA is required for the export of the polysaccharide from the cytoplasm to the periplasm and extracellular fluid. Mutations in *chv*A and *chv*B have been found to be pleiotropic. The mutants lack flagella and are defective in the production and secretion of β-1,2-glucan (Cangelosi *et al.*, 1989). Since other non-pleiotropic flagella-deficient mutants are still virulent, it has been proposed that the lack of periplasmic β-1,2-glucan accounts for the inability of *chv*A and *chv*B mutants to attach and form tumors.

The *exo*C gene encodes a phosphoglucomutase enzyme which is essential for the biosynthesis of UDP-glucose, which is a primary component in the synthesis of numerous polysaccharides. Examination of strains carrying mutations in this gene has revealed that they too are pleiotropic. They are defective in β-1,2-glucan production and the biosynthesis of cellulose and succinoglycan is affected.

Strains with mutations in the *att* loci have also been studied. Phenotypic characterization of these mutations has not revealed any differences with respect to motility, flagella, size of lipopolysaccharides and the production of both β-1,2-glucan and cellulose when compared to the parental strains. However, they were found to lack several outer membrane proteins, but it is not currently known if these specific proteins are involved in attachment (Garfinkel and Nester, 1980; Matthysse, 1987a).

The *cel* gene has also been found to be important in attachment. The *cel* product is important in cellulose biosynthesis and although *cel* mutants still bind and are virulent they do not form aggregates and are easily washed off. These findings suggest that the formation of cellulose is not essential for tumor formation, but is advantageous to the bacteria under certain environmental conditions.

Some time ago it was suggested that there may be specific receptor molecules at the plant cell surface to which the bacteria may bind. This belief is supported by the observations that attachment of the bacteria to the plant cell surface is saturable and tumor formation by virulent strains can be prevented by preinoculating with an avirulent but attaching strain (Neff and Binns, 1985; Lippincott and Lippincott, 1969).

3.1.3.2 Tumor formation

The *vir* genes are essential for tumorgenesis and during infection all the *vir* operons are transcriptionally induced by phenolic compounds and sugars such as galactose, glucose, arabinose, fructose and xylose (Stachel *et al.*, 1986). The VirA protein has a typical leader sequence and a second hydrophobic region that is directly followed by a positively charged sequence that resembles a number of stop transfer sequences in

other membrane spanning proteins (Morel *et al.*, 1990). This transmembrane topology of VirA strongly suggests that it may be an environmental sensor that is able to directly or indirectly recognize phenolic compounds and monosaccharides. VirA also has the ability to undergo autophosphorylation which is believed to be important *in vivo* as it gives VirA the ability to transfer a phosphate group to the highly conserved aspartate residue 52 of VirG. It is this phosphorylation that is thought to alter the ability of VirG to bind *vir* promoters. It has been postulated that the VirG proteins bind to the *vir* boxes located upstream of each *vir* promoter (Steck *et al.*, 1988). The VirG protein has been observed *in vitro* to bind to the *vir* boxes of the *vir*B, E and G promoters of *A. tumefaciens* and the *vir*C and G promoters of *A. rhizogenes*.

For the T-DNA to be transferred to the plant nuclei it must be processed within the bacteria to a transferable form and it must then traverse the bacterial inner and outer membranes, enter the plant cell and reach the nucleus. Following the induction of the *vir* genes molecular reactions occur on the T-DNA element of the Ti plasmid to generate a transferable T-DNA. Single-stranded endonucleolytic cleavages occur at identical positions between the third and fourth bases of the bottom strand of the 25-bp border repeats. These nicks are subsequently used as initiation and termination sites for the displacement of a linear single-stranded copy of the bottom strand of the T-DNA region (Wang *et al.*, 1984). This linear copy is referred to as the T-strand and displacement of this strand starting from the 5′ end of the right border leaves a free 3′ end which can subsequently be used to resynthesize the bottom strand of the T-DNA region.

The VirD1 and D2 proteins are essential for T-strand synthesis (Stachel *et al.*, 1987). Mutations in the genes that encode these proteins lead to ones that are defective

for both nicking at the T-DNA borders and T-strand production. VirD2 is considered to be the protein that guides the T-strands to the nucleus of the plant. Since only the N-terminal 50% of VirD2 is essential for nicking and T-strand formation, it must be this region that recognizes and binds to the 25-bp repeats. The C-terminal end of VirD2 contains a sequence similar to a family of nuclear-targeted proteins of animals and yeasts (Chelsky *et al.*, 1989). If this sequence is removed the virulence is abolished.

The *vir*C locus encodes two polypeptides, VirC1 and VirC2, which enhance T-DNA border nicking. VirC1 binds specifically to what is referred to as the overdrive site (Toro *et al.*, 1989) and mutations in both C proteins attenuate virulence approximately 100-fold. Although the exact functions of these proteins are not known it is possible that since they are not needed for efficient T-strand production they play an ancillary role in DNA unwinding or replacement DNA synthesis.

Once the T-strand has been formed it must traverse the bacterial membrane and peptidoglycan. Since the T-strand must be protected from nucleases during its travel it is quite likely that it exists as a DNA-protein complex and some of the proteins involved are most likely VirE2 and VirD2 (Howard and Citovsky, 1990). The products of the *vir*B gene locus have been proposed to be responsible for the creation of a pore through which the T-DNA complex can pass. Eleven proteins are encoded by *vir*B and at least 10 of these have a hydropathy-profile which suggests an extracellular or membrane-spanning topology (Ward *et al.*, 1988; 1990). The VirB11 protein has been determined to be hydrophilic and has the ability to hydrolyze ATP and autophosphorylate. Therefore, it has been proposed that VirB11 provides the energy required by the T-complex to cross the bacterial membrane. Yusibov *et al.* (1994) have demonstrated that *A. tumefaciens*

transfers single-stranded DNA to the cytoplasm of tobacco cells within 30 min of co-cultivation of the bacteria and that functional *vir*B genes are required for the transfer.

The final step in the genetic transformation of plant cells is the integration of the T-strand into the plant-cell DNA. At the present time very little is known about the fate of the T-DNA in the plant cytoplasm and nucleoplasm. Virts and Gelvin (1985) analyzed the transfer of Ti plasmids from *A. tumefaciens* to *Petunia* protoplasts and found that other Ti plasmid DNA, in addition to the T-DNA, was transferred but that most of it was degraded. Studies on the number and location of T-DNA inserts in mature tumors have shown that one or more T-DNA molecules can be transferred and integrated (Thomashow *et al.*, 1980; Ursic *et al.*, 1983). In those plant cells containing more than one T-DNA copy there does not seem to be a specific way that they are integrated as sometimes they are tightly linked and other times they are dispersed throughout the genome.

The integration events can be described as analogous to illegitimate recombination events involving viral or transfected DNA in mammalian cells. What is believed to happen is that the 5′ end of the T-strand invades a nick within the plant DNA. Short stretches of homology allow partial pairing to occur between both ends of the T-strand and plant DNA. At this point the plant DNA may unwind further thus forming a gap. Any overhangs of the ends of the T-DNA are repaired and ligated to the plant DNA ends (Gheysen *et al.*, 1991). It should be noted that all of those steps only involve one of the two strands of plant DNA and that torsional strain results in the introduction of a nick in the other plant DNA strand. The T-strand is then used as a template for DNA synthesis to form the final integration product.

The T-DNA of several octopine-type and nopaline-type strains of *A. tumefaciens* have been studied and although the genes contained within the T-DNA are not derived from eukaryotes they contain eukaryotic-like regulatory sites. Their promoters contain CAAT and TATA boxes which are typical of plant promoters (Nester *et al.*, 1984) and the mRNA transcribed from these genes contain upstream activating sites that are similar to transcriptional enhancers found in eukaryotes (Ellis *et al.*, 1987). Basically, all of the genes in the T-DNA are involved in either the production of opines or in upsetting the normal balance of phytohormones of the plant cell. Two genes, known as *tms*1 and *tms*2, are involved in the overproduction of auxin (Thomashow *et al.*, 1984). The *tmr* gene has also been determined to be involved in tumor formation. It catalyzes the condensation of isopentenyl pyrophosphate and adenosine monophosphate to isopentenyl-AMP. Isopentenyl-AMP is then converted by plant enzymes to trans-zeatin and trans-ribosylzeatin (Buchmann *et al.*, 1985). The gene *tml* and transcript 5 also play a minor role in tumor formation. Alone these two genes are not oncogenic, but when they are present in combination with the other oncogenes they seem to modulate tumorigenicity (Leemans *et al.*, 1982; Spanier *et al.*, 1989).

3.1.3.3 Suppression of tumorigenicity

Very little work has been published on the inhibition of tumor formation in the *Agrobacterium*-plant interaction. However, *A. tumefaciens* oncogenicity has been found to be suppressed by the presence of a plasmid known as pSa (Farrand *et al.*, 1981; Close and Kado, 1991). The plasmid pSa, originally isolated from *Shigella flexneri*, is conjugative and capable of replication in a wide range of Gram-negative bacteria

(Valentine and Kado, 1989). When pSa is present in *A. tumefaciens* total oncogenic suppression is observed. The presence of this plasmid does not cause any instability of the Ti plasmid and if the strain is cured of pSa then oncogenicity is restored (Farrand *et al.*, 1981).

Johnson and Kado (1988) have demonstrated that the suppressive activity is not related to bacterial auxin production. As well, it has been found that pSa does not alter an exocellular lipopolysaccharide sufficiently to affect the plant cell-binding capability of *A. tumefaciens* (Matthysse, 1987b). It has also been found that this plasmid does not affect the stability or conjugal self-transfer of the resident Ti plasmid. The *vir* genes are also induced in the presence of pSa and the processing of the T-DNA into T intermediates remains operational. Several subclones of pSa have been tested and a 7.6-kb region on the plasmid that confers oncogenic suppression has been identified. The *osa* (oncogenic suppression activity) locus has been mapped and cloned (Close and Kado, 1991). Two ORFs from this region have been identified and studied but the sequences of these two ORFs have not shown any significant similarity to nucleotide or amino acid sequences available in the data bases. One interesting finding was that one of the proteins is rich in hydrophobic residues, and it has been hypothesized that oncogenicity suppression may be effected by an association of this protein with the bacterial membrane which in turn could block T-DNA transfer.

In order to gain further insight into the mechanisms of oncogenic suppression the location of *osa* with respect to its neighboring genes has been studied (Chen and Kado, 1994). It has been found that the *osa* gene is located between the region containing the conjugative transfer genes (*trw* genes) and the region containing the *repA* and *recA* genes.

Upstream of *osa* there are three ORFs . The most distal ORF is 630-bp and encodes a 23.2-kDa protein. The second ORF is the *nuc* gene which encodes a 19.7-kDa Dnase and the third ORF encodes a 22.8-kDa protein whose function is not known. Examination of these ORFs has demonstrated that alone the *osa* is sufficient for completely inhibiting the oncogenicity of *A. tumefaciens* as inhibition is observed when the gene is placed under control of a constitutive promoter. No promoter exists immediately upstream of the coding sequence of *osa,* but deletion analysis has shown that the promoter of the first ORF is required for *osa* transcription. Therefore, *osa* is a part of an operon that consists of at least three other ORFs.

3.1.4 The *Rhizobiaceae* family

Agrobacterium tumefaciens is classified as a member of the *Rhizobiaceae* family. Other genera that are also members of the *Rhizobiaceae* family include *Rhizobium, Bradyrhizobium* and *Azorhizobium.* All species within this family, with the exception of *Agrobacterium radiobacter,* are known to incite cortical hypertrophies on plants. As previously mentioned, the strains of agrobacteria act as tumorigenic phytopathogens that cause gall hypertrophies on the roots and stems of diverse plant species. Unlike agrobacteria, the other genera belonging to this family, collectively referred to as rhizobia, do not act as tumorigenic pathogens but rather share a symbiotic relationship with different leguminous species which result in the formation of specialized root organs known as nodules. It is within these nodules that the bacteria are able to convert atmospheric nitrogen into ammonia. The plants assimilate the ammonia

into amino acids which they use as a source of nitrogen and in return supply the bacteria with organic carbon.

3.1.5 Infection and the formation of nodules by *Rhizobium*

Root infection and the subsequent development of legume root nodules is a multistep process that is generally controlled by a reciprocal signal exchange between the macro- and micro-symbiont. The first step of nodule formation is initiated by preinfection events that occur in the rhizosphere. *Rhizobium* spp. are motile bacteria that respond by positive chemotaxis to the amino acids and dicarboxylic acids present in the plant root exudates as well as to the low concentrations of flavonoids and betaines excreted by the plant (Agular *et al.*, 1988). The involvement of motility in nodule formation has been studied in several species and it has been established that although chemotaxis is not essential for nodulation it does have an influence on competition and establishment in the rhizosphere (Lui *et al.*, 1989).

The next step in nodule formation is the attachment of the bacteria to the root surface. Some investigators have proposed that host specificity is involved in the attachment of *Rhizobium* to the surface of host legume roots and that adherence is mediated by specific binding of particular polysaccharide moieties present on the bacteria to the host plant lectins (Bohlool and Schmidt, 1974). Others, however, have found that host specificity at the attachment level does not exist and that optimal attachment depends on nutrient limitation. Thus, it is the type of limitation that determines whether or not host lectins are involved in the attachment. For example, under carbon-limiting

conditions bacteria adhere to the root in a nonhost specific manner. However, if manganese, oxygen or nitrogen are limited the attachment is via host lectins.

Smit *et al.* (1987) have shown that the attachment of *Rhizobium* to legume root hairs is a two-step process. The first step involves the loose binding of *R. leguminosarum* to single cells on the surface of the root hair. The second step, also known as the cap formation step, involves the accumulation of additional bacteria at the adhesion site thus leading to the formation of bacterial aggregates. Research has indicated that a number of proteinaceous determinants are involved in these two steps, but it is still unclear exactly what type or how many are involved. Two bacterial components involved in the process have been identified and they include rhicadhesion, a proteinaceous adhesion, and cellulose fibrils. The initial binding of *R. leguminosarum* to pea root hair surfaces involves rhicadhesion (Smit *et al.*, 1991). Rhicadhesion activity has been detected in all members of the *Rhizobiaceae* family, however, it is not present in other bacteria such as *E. coli* and *Pseudomonas putida*. A putative plant receptor molecule for rhicadhesion has recently been purified and partially characterized (Swart *et al.*, 1994). It has been demonstrated in the *R. leguminosarum* - pea system that during the second step bacterial cellulose fibrils and the pea lectin Psl are involved in the formation of aggregates (Kijne *et al.*, 1988; Smit *et al.*, 1987). For example, *R. leguminosarum* mutants that overproduce fibrils have been found to increase aggregation whereas cellulose minus mutants do not show formation of aggregates.

Once the bacteria are attached to the surface, root branching, deforming and curling begin to occur (Bhuvaneswari and Solheim, 1985). The substances responsible for this phenomenon in all *Rhizobium* - plant interactions studied to date have been identified

as lipo-oligosaccharides. These lipo-oligosaccharides, also known as Nod factors, are synthesized by some of the *nod* genes.

Purified lipo-oligosaccharides have the ability to induce the deformation of root hairs, the formation of preinfection threads and the division of root cortical cells. Although individual lipo-oligosaccharides have been shown to induce primordium formation most *Rhizobium* species produce a wide variety of lipo-oligosaccharides. Recently it has been demonstrated that co-operative action of lipo-chitin signals are required for the induction of early nodulin, ENOD2, in soybean roots (Spaink, 1994; Minami *et al.*, 1996). Specific modifications of the lipo-oligosaccharides produced by various rhizobia have been found in several cases to determine host specificity. For example, *R. leguminosarium* lipo-oligosaccharides have been observed to elicit the production and secretion of additional flavonoids in the roots of *Vicia sativa* (Recourt *et al.*, 1991). These flavonoids in turn are efficient inducers of additional *nod* genes. The Nod factors also share similar properties with plant hormones and are sometimes regarded as hormone-like molecules.

The chemical structure of these nodulation factors has been determined. The first Nod signal molecule purified was from *R. meliloti* and it was identified as a modified chitin oligomer referred to as a chito-oligosaccharide (Lerouge *et al.*, 1990). The lipo-oligosaccharides are now known to consist of an oligosaccharide backbone of β-1,4-linked N-acetyl-D-glucosamine that varies in size from 3-5 sugar units. A fatty acid group is attached to the nitrogen group of the non-reducing amino sugar moiety and the nature of the fatty acyl chain is variable between the different rhizobial strains. The lipo-oligosaccharide produced by most of rhizobial strains contains fatty acids that are

common constituents of the bacterial phospholipids. Certain strains such as *R. meliloti* and *R. leguminosarium* biovar *viciae* produce lipo-oligosaccharides that contain a multiunsaturated fatty acyl chain (Lerouge *et al.*, 1990). In several of the strains examined a mixture of lipo-oligosaccharides with variations in their fatty acyl moiety are produced.

3.1.6 Biosynthesis of lipo-oligosaccharides

3.1.6.1 UDP-GlcNAc synthesis

The biosynthesis of lipo-oligosaccharides in *Rhizobium* involves some of the *nod* gene products. Many of the initial indications of the function of the Nod proteins came about by comparing their homology to other proteins whose functions were already known (Downie, 1991; Fisher and Long, 1992).

UDP-GlcNAc is believed to be one of the earliest precursors involved in the biosynthesis of the Nod factors. UDP-GlcNAc is also known to be a precursor for the biosynthesis of peptidoglycan and lipid A, both of which are essential for the viability of the bacteria. Since UDP-GlcNAc is essential for both Nod factor and peptidoglycan synthesis it is most likely that rhizobia must produce higher levels of it in order to satisfy UDP-GlcNAc requirement for Nod factor biosynthesis. Such increases in UDP-GlcNAc levels are believed to be due to the *nod*M and *nod*N genes. The *nod*M gene has homology to the *glm*S gene of *E.coli* (Baev *et al.*, 1992; Marie *et al.*, 1992). Therefore, it has been proposed that the NodM protein functions as a fructose-1,6-diphosphate glutamine amidotransferase (GlcN synthase) enzyme (Baev *et al.*, 1991). The predicted biochemical function of the NodM protein has been supported by the observation that the *nod*M gene

is able to complement *E. coli glm*S mutations. Mutations in *nod*M only causes a moderate delay in infection and nodulation (Baev *et al.*, 1991). This observation is consistent with the fact that a mutation in *nod*M results in only a quantitative decrease in the production of lipo-oligosaccharides (Baev *et al.*, 1992). At the present time very little is known about the product of *nod*N and its specific role remains unknown.

The biosynthesis of UDP-GlcNAc also requires a mutase enzyme which is responsible for converting GlcN-6-phosphate to Glc-1-phosphate, a GlcN-1-phosphate N-acetyltransferase and a GlcNAc-1-phosphate uridyl transferase. Therefore, rhizobia most likely contain inducible genes that encode for these enzymes which allows for increased levels of UDP-GlcNAc.

3.1.6.2 Functions of the *nod*A, *nod*B and *nod*C genes

The *nod*ABC genes are known to be a component of a single operon in most *Rhizobium* species. When this set of genes is inactivated their ability to elicit a symbiotic reaction in plants, including root hair curling, formation of the infection threads, cortical cell divisions and nodule formation is lost.

The presence of the *nod*ABC genes has been shown to be sufficient to synthesize the backbone of the signal molecules (Spaink *et al.*, 1991; Geremia *et al.*, 1994) and the functions of each of these three proteins have been well documented. NodC functions, for example, as an N-acetylglucosaminyl transferase (chitin synthase) which creates a chito-oligosaccharide chain. Examination of several different NodC proteins has shown that they all have two predominant hydrophobic domains. A short one is located at the N-terminus and a longer one is near the C-terminus (Torok *et al.*, 1984;

Rossen *et al.*, 1984; Varquez *et al.*, 1991). The NodC sequence has also been determined to have sequence homology to the DG42 protein of *Xenopus lavis*, cellulose synthases and chitin synthase I, II and III (Bulawa, 1992; Atkinson *et al.*, 1992; Debelle *et al.*, 1992; Spaink *et al.*, 1993a). The similarity of NodC to the DG412 and chitin synthases occurs in two regions near the carboxyl terminus. Although the function of the first region is not known the second appears to be responsible for encoding a membrane spanning region. This has been supported by hydropathy plots (Bulawa, 1992). Barny *et al.*, (1993) have identified the presence of the NodC of *R. leguminosarum* exclusively in the inner membrane, and the location and membrane topology of NodC has been further studied by constructing and characterizing fusions of NodC to alkaline phosphatase and β-galactosidase (Barny *et al.*, 1996). In this study it was found that the N-terminal hydrophobic domain of NodC spans the inner membrane in a N_{out}-C_{in} orientation with the large hydrophilic domain being exposed to the cytoplasm. This orientation, however, appears to be dependent upon the presence of the C-terminus hydrophobic region. From this research it can be concluded that NodC is located in the inner membrane with the majority of the protein in the cytoplasm. This location for NodC, therefore, allows the synthesis of the chitin oligomer on the inner surface of the cytoplasmic membrane. Since NodA and NodB are located in the cytoplasm (Johnson *et al.*, 1989) this would be a perfect location for NodC, as NodA and NodB would have access to the terminal end of the oligomer for further modification.

The synthesis of most oligosaccharides usually occurs by the transfer to a sugar residue which is glycosidically linked to a lipid carrier. For example, the biosynthesis of peptidoglycan, the bacterial polysaccharide most similar to chitin, is initiated on an

undecaprenol phosphate carrier lipid. In Crustacea the synthesis of lipid-linked chitin oligosaccharide occurs on a polyprenol intermediate. Prior to extension by the sequential addition of more GlcNAc residues the oligosaccharide is transferred to a protein acceptor (Horst, 1985). A monoglycoslated diacylglycerolipid is believed to be the lipid primer for chitin synthesis in the zoospores of *Blastocladiella emersonii* (Mills *et al.*, 1980). Glycosyldiacylglycerol glycolipids have also been reported in *Spirochaetes*, pseudomonads, *Bacteroides* and *Mycoplasma* (Kates, 1990). Such lipids, however, are rather uncommon in Gram negative bacteria. In *Rhizobium* it is believed that glycosyldiacylglycerol molecules may serve as the lipid anchor in the synthesis of the Nod factors. This is based on the fact that such molecules are present in *Rhizobium* and that glycerol has been found as the aglycone moiety of certain Nod factors. It is also possible that different species of *Rhizobiaceae* have different anchors.

Prior to the attachment of the correct fatty acyl chain to the nonreducing N-acetylglucosamine residue of the backbone, the corresponding N-acetyl amino group must be deacetylated. NodB is responsible for deacetylating the non-reducing N-acetylglucosamine residue. Examination of the NodB has revealed that it is capable of deacetylating the terminal GlcNAc residue of chitotetrose, chitotriose and chitobiose. It is not able, however, to deacetylate monomeric GlcNAc (John *et al.*, 1993). This is a very interesting finding as the NodB sequence shares homology with a chitin de-acetylase isolated from the fungus *Mucor rouxii* which requires a substrate of at least four GlcNAc residues for activity (Kafetzopoulos *et al.*, 1993). It has also been found to have sequence similarity to an open frame from *Bacillus stearothermophilus* which is thought to encode an enzyme that de-acetylates peptidoglycan (Katetzopoulos *et al.*, 1993).

Although these reports imply that NodB de-acetylates a terminal GlcNAc residue during the synthesis of Nod factors the actual *in vivo* substrate has not yet been determined. Thin-layer chromatography analysis of water soluble GlcN metabolites produced by a *nod*B mutant of *R. leguminosarum* biovar *viciae* indicates the presence of chitotriose to chitopentose oligomers (Spaink *et al.*, 1993a). Thus, NodB most likely uses the oligomers as substrates.

The NodA protein is involved in completing the backbone structure by attaching an acyl chain to the free amino group. Rohrig *et al.* (1994) have reported that cell extracts of *R. meliloti* expressing the *nod*A gene are capable of acylating β-GlcN-(1-4)-[β-GlcNAc]$_2$-(1-4)-β-GlcNAc while extracts of *nod*A mutants are not able to do so (John *et al.*, 1993).

3.1.6.3 *Nod* genes involved in modifying lipo-oligosaccharides.

The *nod*E and nodF genes also play an important role in the production of lipo-oligosaccharides. Examination of the sequences of NodE and NodF have shown that NodF shares homology with the acyl carrier proteins and NodE shares homology with various β-ketoacylsynthases. These observations have led to the hypothesis that the gene products function in the synthesis of the highly unsaturated lipid moiety (Spaink *et al.*, 1989). This hypothesis has been further supported by the observation that the NodF contains a 4' phosphopantetheine prosthetic group which can function as a carrier for acyl chains during acid biosynthesis (Geiger *et al.*, 1991). As mentioned, *R. meliloti* and *R. leguminosarium* biovar *vicia* normally secrete Nod factors with a highly unsaturated fatty acid. However, the *nod*E mutants of these strains secrete metabolites that are N-acylated

by vaccenic acid. Thus, NodE and NodF are unique to those *Rhizobium* species that contain multiple unsaturated fatty acids in their Nod factors.

Several genes have been found to be involved in the addition of substituents to the basic lipo-oligosaccharide structure. These genes include the *nod*P, *nod*Q, *nod*H and *nod*L. Studies undertaken on the *nod*H, *nod*P and *nod*Q genes, which are present only in *R. meliloti*, have revealed some interesting findings. When the *nod*H gene is mutated absence of the sulfonation of all lipo-oligosaccharides is observed. Upon mutating the *nod*P and Q genes a mixture of sulphonated and nonsulphonated Nod factors are produced (Roche *et al.*, 1991a). The reason why such a leaky phenotype is observed in *R. meliloti* is that there is a second copy of the *nod*PQ genes present on a second mega plasmid. Should this second set of genes be mutated sulfated factors are not produced. In Gram negative bacteria it is known that the first steps of sulfate metabolism involve the synthesis of activated forms of sulfate derived from ATP by an ATP sulfurylase and an APS kinase. NodP and a portion of NodQ exhibit strong homology to CysD and CysN, which are subunits of an ATP sulfurylase of *E. coli*. Another region of *nod*Q is also homologous to CysC which is a known APS kinase. Schwedock and Long (1989) have demonstrated that the NodP and NodQ have ATP sulfurylase and APS kinase activity *in vitro* and that their function is most likely in the synthesis of 3′ phosphadenosine 5′-phosphosulfate. Finally, it has also been documented that NodH shares homology with sulfotransferases which would be consistent with its role in the addition of the sulfate moiety (Roche *et al.*, 1991a). The role of the NodL protein has been studied and it appears to be a transacetylase that is involved in the addition of the O-acetyl moiety. It appears as if NodL uses the complete unacetylated lipo-oligosaccharides as a substrate.

Some of the Nod factor modifications that occur seem to restrict the symbiotic host-range while others extend it. Modifications such as sulfation at O-6 of the reducing GlcNAc residue and the presence of multiple unsaturated fatty N-acyl substituent in *R. meliloti* are examples of modifications that restrict host-range (Lerouge *et al.*, 1990; Roche *et al.*, 1991b). Others, such as MeFuc at O-6 of the reducing GlcNAc from *R. fredii* and *B. japonicum*, extend the host-range.

Staehelin and co-workers (1994) have put forth a possible mechanism for the host-specific effects of the numerous factors. They found that in the case of *R. meliloti* endo-chitinases from alfalfa, the host plant, rapidly degraded several Nod factors except for the alfalfa-specific molecule. Similar findings with Nod factors from *B. japonicum* and *Rhizobium* NGR234 have also been observed. Reports such as these have led to the proposal that host specific structural modifications of the various Nod factors work to protect them from degradation by the host plant chitinases.

3.1.6.4 Control of nodule induction

Nodule development is governed by signal exchange between the bacteria and the host plants. As previously mentioned, the first signal in the symbiotic pathway comes from the plant which induces the expression of the *nod* genes in conjunction with the constitutively produced activator protein NodD. These flavonoids and isoflavones are products of the phenyl-propanoid pathway and several may act as *nod* gene inducers at varying efficiencies. There are also flavonoids produced by the plant roots that do not affect *nod* gene expression or act as inhibitors. These findings suggest that *nod* gene

activation is controlled by a critical ratio of inducing and inhibitory molecules (Kondorosi, 1991).

The early studies of Allen and co-workers (1953) indicated that nodule-like structures can appear on roots by simply changing the hormonal balance within the root, similar to the formation of galls on dicots infected with *A. tumefaciens*. As well, studies undertaken on the correlation between the hormonal balance and nodulation of *Medicago* root cells with auxin-transport inhibitors have clearly shown that they can induce the formation of empty nodule-like structures (Hirsch *et al.*, 1989). This suggests that plants contain the essential components of the nodule development pathway, and that these pathways are most likely activated by a change in the hormonal balance. Nodulation kinetics of different *Medicago saria* lines which vary in auxin-sensitivity have been studied and it was determined that a direct correlation exists between the frequency of spontaneous nodulation and auxin sensitivity (Kondorosi *et al.*, 1993), the highest auxin-sensitivity exhibiting the highest frequency of spontaneous nodule formation.

Research undertaken on *Agrobacterium rhizogenes*, which is the causative agent of hairy root disease, has shown that the *rol*ABC genes alter the auxin-sensitivity and endogenous active hormone ratios (Shen *et al.*, 1988; Estruch *et al.*, 1991). To test further if changes in hormonal balance are involved in the nodule induction, different combinations of these genes were transferred to two lines of *Medicago*. The nodulation ability of the transformed plants were definitely affected as those containing *rol*ABC or *rol*B nodulated much faster than the control plants (Kondorosi et al., 1993). As well, the number of nodules present on the transgenic plants was much greater. These and the

previous results suggest that the mode of action of Nod signals is probably connected to the signal transduction pathways of plant hormones.

3.1.6.5 Export of the Nod factors out of the bacterial cell

Although very little is currently known about the export of Nod factors out of the bacterial cell it is believed that NodI and NodJ play a role in the export. These genes are present in *R. leguminosarum* biovar *trifolii*, *B. japonicum*, *R. etli* and *Azorhizobium caulinodans* (Evans and Downie, 1986; Geelen *et al.*, 1993; Vazquez *et al.*, 1993) and reside downstream of *nod*C. Thus, they appear to be part of the same operon as *nod*ABC.

Upon examination of the *nod*I and *nod*J mutants of *R. leguminosarum* it was found that they retained the ability to synthesize the Nod factors, but that they failed to export them (McKay and Djordjevic, 1993). The location of these two proteins has been determined to be the cytoplasmic membrane, which is consistent with their proposed function (Schlaman *et al.*, 1990). Examination of the sequences of NodI and NodJ has shown that they are similar to the KspT and KspM proteins of *E. coli* which make up a transport-system that exports the Kdo-containing capsular antigen across the cytoplasmic membrane (Vasquez *et al.*, 1993; Smith *et al.*, 1990). Analogous Kdo-rich capsular antigens have been isolated from *R. fredii* and *R. meliloti* that can functionally substitute for *R. meliloti* extracellular polysaccharides in the symbiotic infection of alfalfa (Reuhs *et al.*, 1993). So far no studies have been published on *nod*I and *nod*J mutants that are defective in the export of capsular antigen, but the presence of these transport genes could account for why they are not completely defective in nodulation.

The exact mechanism by which NodI and NodJ export the Nod factors out of the bacteria is not yet fully known. Since NodI and Nod J are similar to KspM and KspI, which are believed to be involved in the transport of capsular antigens, it has been postulated that NodI and NodJ provide an export mechanism. (Carlson *et al.*, 1994). It has been proposed that a Nod factor is synthesized on the inner surface of the cytoplasmic membrane using a glycone carrier. The lipo-oligosaccharide is transported through the membrane in to the periplasm by NodI and NodJ and secreted to the surface by an unknown protein which could be analogous to the periplasm protein KspD, whose protein sequence is similar to the ExoF protein of *R. meliloti* which is involved in exopolysaccharide export (Reuber and Walker, 1993; Glucksmann *et al.*, 1993). There are two theories as to how the Nod factor is released from the cell. One is that as the Nod factor is being transferred across the cytoplasmic membrane it may be transferred from a undecaprenol phosphate carrier lipid to a lipid anchor that is targeted to the outer membrane. On the other hand, the factor may simply be released extracellularly from the carrier during transport.

3.1.6.6 Lipo-oligosaccharides and their possible role as endogenous signal molecules in plants

It is well known that lipo-oligosaccharides have various effects on plant morphogenesis. Due to the fact that the rhizobial signals resemble chitin, and since it has been demonstrated that chitinases are involved in plant embryogenesis (De Jong *et al.*, 1993), it has been proposed that similar molecules may be present in uninfected plants.

Spaink and co-workers (1993b) believe that this hypothesis could explain why various classes of chitinases are constitutively expressed in the pistils of flowers.

Benhamou and Asselin (1989) found that there is an abundance of N-acetyl-D-glucosamine residues in the secondary walls of plants. Why these residues are present in the plants is not exactly known, but Schmidt *et al.* (1991) discovered that transgenic plants containing the *nod*A and *nod*B genes of *R. meliloti* are disturbed in their normal development. Spaink *et al.* (1993b) postulated that this disruption could be due to interference with the synthesis of hypothetical chitin derivatives. They also suggested that since there is a high level of similarity of the primary and secondary structure of NodC and the DG42 protein of *Xenopus leavis* (Bulawa, 1992; Sandal and Marcker, 1990; Semino and Robbins, 1995) the chitin-like molecules may also be involved as signals in vertebrate animals.

Spaink and co-workers (1993b) tested their hypothesis by performing radioactive labelling experiments with uninfected *Lathyrus* plants which were incubated in a solution of ^{14}C-labelled acetate and then extracted with n-butanol. The extracts were then analyzed using TLC systems that are known to separate Nod metabolites. The results showed that flowering *Lathyrus* plants contain many lipophilic compounds which migrate similarly to the rhizobial lipo-oligosaccharides on TLC plates. To determine whether compounds related to chitin were present in the extracts they were incubated with a chitinase which degrades the rhizobial lipo-oligosaccharides. After such treatment two spots were found to migrate very similarly to the lipo-oligosaccharide signals of *R. leguminosarium* biovar *viciae* which suggests that lipophilic chitin derivatives are present in uninfected plants. The exact role they play is still unknown.

3.1.6.7 Putative receptors for Nod factors

A number of researchers have established that Nod factors induce deformation of root hairs at nano- to picomolar concentrations (Lerouge *et al.*, 1990; Spaink *et al.*, 1991). It has also been demonstrated that these changes are only induced by acyl-oligochitin factors and not by chitin oligomers (Heidstra *et al.*, 1994). By using molecular markers of the cell cycle and *in situ* hybridization in the cortex, it has been shown that rhizobial infection re-activates the cell cycle in the cortical cells (Yang *et al* ., 1994). The cells of the infection thread, however, are arrested in the G2 phase of the cycle. Therefore, it appears as if the role of the Nod factors is to activate the cell cycle machinery of the cortical cells. For these changes to occur there must be some type of receptor molecule or molecules that interact with the acyl-oligochitin factors.

It is most likely that the acyl-oligochitin factors are transformed prior to interacting with the plant cell machinery. This is supported by the fact that when the Nod factors are near the root of a plant they are degraded by chitinases which are produced by the plant. Although it is not likely that these factors represent activated metabolites it is possible that they are active within the plant tissue. The receptors involved in this reaction are probably very sensitive and specific as delivery of specific factors at very low concentrations by spot inoculation induces selective responses (Roche *et al.*, 1991b). However, studies suggest that the presence of more than one type of receptor would explain the different plant responses that are observed. For example, although *nod*FE and *nod*L mutants of *R. meliloti* were able to cause root hair deformations they were poor in initiating curling and infection thread formation (Ardourel *et al.*, 1994). These Nod

factors either lacked a fatty acyl group with a conjugated enoyl group or an acyl group on the nonreducing glucosaminyl end. These findings suggest that the uncoupling of these responses is related to the different requirements of the Nod factors and it has been proposed by Ardourel *et al.*, (1994) that there are two types of Nod factor receptors. The first are probably entry receptors that have stringent structural requirements such as a sulphate group, C16:2 fatty acid or O-acetyl group, and would function during the initial bacterial ingestion in the growing root hairs. The second are probably receptors that are responsible for signaling within the plant during the course of infection in the epidermal and cortical cells and would have less stringent requirements.

3.1.7 Chitinases

Of the numerous defense-related proteins produced by plants the production and accumulation of the hydrolytic enzyme chitinase has been the most extensively studied, from which it has been concluded that chitinases is a true pathogenesis-related (PR) protein (Graham and Graham, 1991). Chitinase catalyzes hydrolysis of the β-1,4 linkages of the N-acetyl-D-glucosamine (GlcNAc) polymer chitin to oligomers and monomers (Muzzarelli, 1977). The complete hydrolysis of the polymer chitin to GlcNAc is carried out by a chitinolytic system that consists of at least two hydrolases (Jeuniaux, 1966). Chitinase (poly-β-1,4 (2-acetamido-2 deoxy) -D-glucoside glycanohydrolase: EN 3.2.1.14), also referred to as endochitinase, hydrolyzes chitin to oligomers and dimers. The products formed by the action of endochitinases are subsequently degraded to monomers by chitobiase (chitobiose acetamidodeoxy-glucohydrolase: EN 3.2.1.29) (Muzzarelli, 1977; Kless *et al.*, 1989).

Chitinase genes have been isolated and studied from a number of different organisms including plants, bacteria, insects and fungi (Nitzche, 1983; Boller, 1985; Clarke and Tracey, 1956; Jeuniaux, 1966). Chitinases isolated and studied from plants and bacteria have been determined to differ markedly in their antifungal activity. This difference is believed to be related to the differences in substrate specificity between the two classes of chitinases which include endochitinases and exochitinases (Roberts and Selitrennikoff, 1988). For example, most of the plant chitinases studied function as endochitinases and often contain lysozyme activity. Endochitinases tend to have high antifungal activity due to the fact that they have the ability to cleave the β-1,4 linkages of chitin regardless of its size.

3.1.7.1 Occurrence of chitinases in nature

Chitinases are produced by a wide variety of organisms including bacteria (Clarke and Tracey, 1956), nematodes (Jeuniaux, 1966), fungi (Bartnicki-Garcia, 1968), insects (Koga *et al.*, 1983) and higher plants (Abeles *et al.*, 1970; Boller *et al.*, 1983). Since chitinase activity occurs in all species which contain the substrate chitin, it is considered to have a role in the growth, development and differentiation of the chitin-containing organisms. In bacteria, chitinases tend to play a nutritional role as digestion of chitin leads to availability of an abundant source of carbon, nitrogen and energy for use by the bacteria. These chitinases are considered to be important from an ecological viewpoint since they are involved in the removal and recycling of chitin, the second most abundant biopolymer produced on the continents and in the oceans. The chitinases produced by fungi are responsible for the softening or lysis of cell walls which is

necessary for growth, initiation of hyphal branches and cell separation (Reyes *et al.*, 1988). In entomopathogenic fungi chitinases aid in the infection and degradation of insect cuticles (Khachatourians, 1991). It is also believed that insects produce chitinases in order to carry out a role similar to that of fungi. Since insects are known to recycle approximately 90% of their old cuticle during molting it has been postulated that chitinases are responsible for the degradation of the chitin which is a major component of their cuticle (Mommsen, 1980; Chen *et al.*, 1982).

Chitinases have been isolated from several different plant species such as bean, soybean, wheat, barley, tobacco, melon, tomato and pea (Boller *et al.*, 1983; Shinshi *et al.*, 1987; Nasser *et al.*, 1990; Kragh *et al.*, 1990; Jacobsen *et al.*, 1990; Leah *et al.*, 1991; Chang *et al.*, 1995). Although all higher plants tested to date have been found to produce chitinase, they lack its specific substrate chitin. Thus, it has been suggested that the chitinase produced by plants may be the remnant of an enzyme that lost its original role during the course of evolution (Jeuniaux, 1966) or that it is a defense protein that acts against chitin-containing pathogens (Schlumbaum *et al.*, 1986). Recently, it has been shown that chitinase expression is also under developmental control in certain organs and tissues and appears to have an important function in early embryo development (De Jong *et al.*, 1992; Neale *et al.*, 1990). Plant chitinases have also been observed to inactivate the lipo-oligosaccharide signal molecules produced by certain *Rhizobium* strains. Therefore, it has been suggested that chitinases play a role in helping to determine host specificity in the symbiosis process (Roche *et al.*, 1991b).

3.1.8 Plant Chitinases

3.1.8.1 Classification and structure of chitinases

Amino acid sequences have been published from at least 29 plant chitinases (Table 3.1). A comparison of these sequences has shown that plant chitinases can be subdivided into two major structural groups. The first group includes proteins that are related to the basic chitinases of tobacco and bean, and are conserved at 43% of the positions in the catalytic domain. The second group includes chitinases that are highly conserved to one another. For example, chitinases belonging to the second group have identical amino acids at 48% of the positions. Further studies undertaken on the primary structure of the first group of plant chitinases has led to the subdivision of the group into three distinct classes, Class I, II and III (Shinshi *et al.*, 1990).

Class I plant chitinases contain a N-terminal cysteine-rich domain of approximately 40 amino acids and a highly conserved main structure which is separated by a variable hinge region and a C-terminal extension (Shinshi *et al.*, 1987, 1990). A closer examination of the conserved region starting at the N-terminal end of the mature proteins has revealed that the 40 conserved amino acids include 8 cysteines. Of interest is that this specific domain is also present in other proteins that do not exhibit chitinase-like activity. For example, a number of lectins that have been isolated from wheat, rice, barley and stinging nettle (Wright and Raikhel, 1989; Wilkens and Raikhel, 1989; Lerner and Raikhel, 1989; Broekaert *et al.*, 1989) that contains this specific domain. This cysteine-rich portion of the protein has also been identified in two potato wound-induced proteins whose exact functions are not currently known (Stanford *et al.*, 1989) and

Table 3.1 Plant chitinases that have been isolated or cloned

Plant	Class	Reference
Onion	-	Van Damme *et al.* (1993)
Arabidopsis thaliana	II, III	Samac *et al.* (1990)
Azuki bean	III	Ishige *et al.* (1991)
Barley	Ib, II	Swegle *et al.* (1989)
		Leah *et al.* (1991)
Bean	Ia, III, IV	Broglie *et al.* (1986)
		Hedrick *et al.* (1988)
		Margis-Pinheiro *et al.* (1991)
Canola	IV	Rasmussen *et al.* (1992)
Carrot	-	Kurosaki *et al.* (1989)
Cucumber	III	Metraux *et al.* (1989)
Corn	-	Nasser *et al.* (1988)
Job's tears	I	Ary *et al.* (1989)
Melon	I	Roby and Esquerre-Tugaye (1987)
Oat	II	Fink *et al.* (1988)
Vine	III	Bernasconi *et al.* (1987)
Pea	Ib	Vad (1991)
Peanut	-	Herget *et al.* (1990)
Petunia	II	Linhorst *et al.* (1990)
Poplar	Ia	Parsons *et al.* (1989)
		Davis *et al.* (1991)
Potato	Ia	Gaynor (1988)
		Laflamme and Roxby (1989)
Pumpkin	I	Esaka *et al.* (1990)
Rice	Ia	Huang *et al.* (1991)
Soybean	-	Wadsworth and Zikakis (1984)
Sugar beet	III, IV	Mikkelsen *et al.* (1992)
Thornapple	-	Broekaert *et al.* (1988)
Tobacco	Ia, II, III, IV	Shinshi *et al.* (1987, 1990)
		Van Buuren *et al.* (1992)
		Fukuda *et al.* (1991)
		Linhorst *et al.* (1990)
		Payne *et al.* (1990)
		Lawton *et al.* (1992)
Tomato	I, II	Breijo (1990)
Elm	-	Hajela *et al.* (1992)
Wheat	I	Molano *et al.* (1979)
Yam	-	Tsukamoto *et al.* (1984)

in the hevein protein of *Hevea brasiliensis* (Lucas *et al.*, 1985). Examination of the cDNA clone of hevein has revealed that it encodes a larger protein with a region that is highly homologous at its N-terminal.This region is highly homologous to the potato proteins just described. Recently, more proteins which are implicated in plant defense have been reported to contain similar cysteine-rich modules. Thionins contain cysteine-rich modules that are of similar length with a similar spacing of the cysteines (Garcia-Olmedo *et al.*, 1992). Very small proteins with antimicrobial activity have also been identified with cysteine-rich regions (Cammue *et al.*, 1992; Terras *et al.*, 1992; Terras *et al.*, 1993; Terras *et al.*, 1995). Although these regions are not highly conserved with the Class I chitinases it is very likely that these cysteine-rich regions are important to the antimicrobial activity of the proteins.

Between the cysteine-rich domain and the remainder of the Class I chitinases is a hypervariable region. It is believed that this region functions as a spacer that joins the cysteine-rich module to the main portion of the protein. This region has been determined to range from 2 to 18 or more amino acids (Shinshi *et al.*, 1990; Laflamme and Roxby, 1989). In some cases, such as the poplar enzyme WIN-8, the region is absent (Parsons *et al.*, 1989). The main structure of the Class I chitinases has been found to be conserved at approximately 54% of the positions when the sequences of a number of proteins from different species are aligned. This conserved main structure is the catalytic domain of the enzyme.

Class II chitinases have been classified as enzymes that are acidic proteins which are located in the extracellular spaces of the plant. This class of chitinases have been found to be synthesized as preproteins containing a hydrophobic signal peptide (Payne *et*

al., 1990; Linthorst *et al.*, 1990). The major structural differences between these chitinases and those belonging to Class I are that they do not contain the N-terminal cysteine-rich domain, the spacer, the variable region of the catalytic domain and the C-terminal extension. However, the catalytic domains of these two classes of chitinases are highly conserved (Collinge *et al.*, 1993).

The Class III chitinases show no sequence similarity to the enzymes in Class I or Class II. This group of chitinases include lysozyme/chitinases and chitinases from such plants as *Hevea, Rubus* and *Arabidopsis* (Boller *et al.*, 1988; Samac *et al.*, 1990). The enzymes that fall into this group have highly conserved sequences amongst themselves. It should be noted, however, that some plants such as *Arabidopsis* and bean produce a number of chitinases that fall into either Class I or II, as well as, Class III (Samac *et al.*, 1990; Margis-Penheire *et al.*, 1991). The Class III plant chitinases have been shown to have a weak similarity to bacterial chitinases and hydrophobic cluster analysis has been used to deduce a putative active site that contains four aspartic or glutamic acid residues (Henrissat, 1990).

A fourth class of chitinases which would include the rapeseed chitinase ChB4 (Rasmussen *et al.*, 1992a, 1992b), acidic bean PR4 chitinase (Margis-Pinheiro *et al.*, 1991) and basic sugar beet chitinase IV (Mikkelsen *et al.*, 1992) has been proposed by Collinge *et al.* (1993). The chitinases that are a part of this proposed Class contain a cysteine-rich domain and a conserved main structure that resemble those of the Class I chitinases. Due to four deletions, however, they are significantly smaller than the Class I chitinases. Comparison between the sequences of the Class I and Class IV chitinases has revealed that there is only a 41-47% identity compared with approximately 63% identity

between the individual Class IV chitinases. As well, the Class I and IV chitinases are serologically distinguishable (Mikkelsen *et al.*, 1992).

It is believed that the N-terminal cysteine-rich domain present in the various classes of plant chitinases is a chitin-binding domain. Such domains are apparent in other proteins such as wound-induced WIN proteins, the stinging nettle lectin and the wheat germ agglutinin (Broekaert *et al.*, 1989; Lucas *et al.*, 1985; Stanford *et al.*, 1989). It is possible that these cysteine-rich proteins evolved as a result of a gene fusion of the chitin-binding domains with chitinase domains (Chrispeels and Raikel, 1991).

3.1.8.2 Regulation of plant chitinases by ethylene

The possibility that chitinases are involved in the defense of plants against pathogens was first proposed by Abeles and co-workers in 1970 (Abeles and Florence, 1970; Abeles *et al.*, 1970) who found that chitinase, along with β-1,3-glucanase, was coordinately induced by exogenous ethylene. Since it had already been demonstrated that the cell walls of fungi contain significant amounts of β-1,3 glucans and chitin, and because there was no information suggesting that higher plants contained chitin, they postulated that these enzymes may play a role in protecting plants from fungal pathogens. They also hypothesized that ethylene, which is synthesized endogenously in response to infection, may also induce the enzymes and therefore enhance the defense potential of the plants. Support for the hypothesis put forth by Abeles *et al.* (1970) has been obtained by several research groups who have examined the induction of chitinase and its significance in the defense of various plant species against invading pathogens (Boller, 1985; Schlumbaum *et al.*, 1986; Broglie *et al.*, 1991; Graham and Graham, 1990).

In the 1980's the effect of ethylene on the induction of chitinase in bean leaves was investigated (Boller *et al.*, 1983: Boller, 1985). It was found that chitinase activity in bean leaves begins to increase within 6 hours after the onset of treatment with ethylene and that it is induced 30-fold within 24 hours of treatment. Upon treating the leaves with 1-aminocyclopropane-1-carboxylic acid (ACC), the biosynthetic precursor of ethylene, endogenous ethylene production occurred and chitinase production was induced. It has also been demonstrated that the induction of chitinase can be halted, even after it has begun, by withdrawing the ethylene. Thus, it can be concluded that ethylene does induce chitinase production in certain plants. The finding of ethylene production in plants by pathogenic infection and its correlation with the synthesis of chitinase has been confirmed by *in vivo* labelling and immunological techniques (Boller, 1985). In that study it was found that following treatment with ethylene chitinase is one of the most abundant polypeptides synthesized in bean leaves. Several cDNA clones encoding chitinase were isolated, characterized and used to monitor mRNA levels following ethylene treatment so that its role in the regulation of gene expression could be studied. The results from these experiments indicated that the induction of chitinase by ethylene in bean leaves was accompanied by a marked increase in the steady-state chitinase mRNA levels. These results provided the evidence needed to conclude that the effect that ethylene has on chitinase gene expression in bean plants is at the transcriptional level and not at the post transcriptional level (Broglie *et al.*, 1986; Broglie *et al.*, 1989). Ethylene has also been shown to induce chitinase expression in melon plants and pea roots (Roby *et al.*, 1985; Roby *et al.*, 1986). However, the role of ethylene may be different in specific species as it was found not to induce chitinase expression in soybean roots (Vigniotelli, 1991).

Furthermore, ethylene-independent induction has been demonstrated in tobacco and pea plants (Lotan and Fluhr, 1990; Mauch *et al.*, 1984).

3.1.8.3 Induction of plant chitinases by pathogen-induced stress

Measurements of steady-state mRNA levels have indicated that expression of plant chitinases is induced in response to fungal infection and treatment with fungal elicitors, and in the case of cucumber and tobacco infection with viral agents (Pegg and Young, 1982; Chappell *et al.*, 1984; Roby and Esquerre-Tugaye, 1987). Several studies have been undertaken to examine directly the antifungal activities of chitinases. Schlumbaum *et al.*, (1986), for example, examined the effect purified bean chitinase has on the growth of the fungus *Trichoderma viride*. In these experiments a marked concentration-dependent inhibition of fungal growth around wells that contained bean chitinase was observed. When the bean chitinase was boiled, however, no inhibition of fungal growth was observed. As well, antibodies raised against the purified bean chitinase were found to block chitinase activity and prevent the inhibitory effect of the chitinase on fungal growth. When the bean leaves were infected with *Colletotrichum lindemuthiam* and *Uromyces phaseoli* the level of chitinase increased approximately ten-fold compared to the levels observed in uninfected leaves (Schlumbaum *et al.*, 1986; Hedrick *et al.*, 1988).

In melon plants, the level of translatable chitinase mRNAs also increased following infection by the fungal pathogen *Colletotrichum lagenarium* (Roby and Esquerre-Tugaye, 1987). In this case, chitinase activity was observed to increase throughout the plant when hypocotyls were locally infected with the fungal pathogen. As

well, treatment with a fungal elicitor also gave rise to increased levels of chitinase activity. When the elicitor-treated plants were exposed to *C. lagenarium* an increase in resistance to infection was observed. These results indicate that elevated levels of chitinase are responsible, at least in part, for induced resistance to *C. lagenarium* (Roby *et al.*, 1988).

Differential regulation of chitinase isozymes from pea, tobacco, turnip, barley and cell cultures of peanut has been reported. When pea plants were challenged with fungal pathogens at least two chitinases were found to show differential regulation (Mauch *et al.*, 1988; Vad *et al.*, 1991). Kragh and co-workers (1990) have shown that different chitinase isoforms are found in barley leaves and grain, but only one out of five basic chitinases is induced when the leaves are infected with *Erysiphe graminis*. Similar findings have been observed in peanut cell cultures (Herget *et al.*, 1990) where, upon elicitor treatment, only the mRNA of one chitinase form is induced.

Chitinase activity has been found to be higher in resistant cultivars during the early stages following inoculation than in susceptible cultivars in most of the plant-fungi interactions studied. This has been found in bean inoculated with *Colletotrichum lindemuthianum* (Daugrois *et al.*, 1990), canola infected with *Phoma lingam* (Rasmussen *et al.*, 1992a, 1992b) and tomato inoculated with *Cladosporium fulvum* (Joosten and de Wit, 1989). In *Brassica campestrus* Conrads-Strauch *et al.* (1990) found that two different isozymes, of the same size, are differentially induced in response to virulent and avirulent pathovars of *Xanthomonas campestris*. Chitinase, along with other PR proteins, are also induced in symbiotic interactions. For example, chitinase levels increase in *Allium porrum* roots when infected by mycorrhiza and in soybean nodules infected with

Bradyrhizobium japonicum (Spanu *et al.*, 1989; Staehelin *et al.*,1992). These results are interesting as the induced chitinase levels may reflect the need to protect the host from external pathogenic invasion during symbiosis or to prevent pathogenic development of the symbiont.

Treatment of plants with elicitors also induces chitinase activity. Hedrick *et al.* (1988) carried out a number of experiments which demonstrated that the synthesis of chitinase is stimulated in bean cell suspension cultures when treated with fungal elicitors. A very rapid activation of chitinase transcription was observed in the treated bean cell cultures. A ten-fold increase was observed 5 min after treatment and a 30-fold increase was observed after 30 min. Maximum chitinase levels were reached approximately 2 h after treatment. This rapid induction strongly suggests that the signal transduction system is in place prior to elicitor treatment and that there are few intervening steps between the elicitor binding to a putative cell receptor and the specific transcriptional activation of the chitinase gene. A significant increase in chitinase activity was also observed when cultures of parsley cells (*Petroselinum crispum*) were treated with cell wall fragments of *Phytophthora megasperma* f. sp. *glycinea* (Kombrink and Hahlbrook, 1986). Within 12 h of treatment with cell wall fragments a ten-fold increase in chitinase activity was observed (Broglie, 1985).

Similar findings have been observed in cultured carrot cells when they are incubated with insoluble mycelial walls of *Chaetomium globosum* (Kurosaki *et al.*, 1986). The results of these experiments also suggest that the induced chitinase hydrolyzes the fungal walls and the soluble fragments liberated from the hydrolysis of the walls stimulate the biosynthesis of phenolic acids. This is interesting as phenolic acids are

precursors for the synthesis of lignin which is involved in forming a barrier to the invasion or spread of microorganisms. Chitinases may thus play a dual role in disease resistance.

3.1.8.4 Promoter regions of plant chitinase genes

It has been well established that the regulation of transcription of many genes depends upon the presence of *cis*-acting elements in the 5′ flanking region of the coding sequence (Benfrey and Chua, 1989). Steady-state levels of chitinase mRNA have been measured and the results indicate that chitinases are generally regulated at the level of transcription. In order to study the development and inducible pattern of expression of various chitinase genes, as well as possible important *cis*-acting elements, several research groups have transformed various plants with different chitinase promoters that are fused to reporter genes (Roby *et al.*, 1991; Samac and Shah, 1993; Kellmann *et al.*, 1996).

The induction of the bean chitinase 5B promoter in transgenic tobacco plants has been studied to determine how this gene is activated upon transfer to other plants. In this study a 1.7-kb fragment bearing the 5B upstream sequences of the bean chitinase gene was fused to the coding region of the *E. coli* β-glucuronidase (GUS) gene (Broglie *et al.*, 1989; Broglie *et al.*, 1993), The chimeric reporter gene was introduced into plants which were then treated with ethylene. After ethylene treatment the induction of GUS activity was observed, thus indicating that the sequence information necessary for ethylene expression was contained within this region of DNA (Broglie *et al.*, 1989). A significant induction of GUS enzyme activity was also observed when these plants were infected

with fungal pathogens. Assays of the tissue infected with *Botrytis* have indicated that although GUS activity was preferentially localized at the site of inoculation, a slightly weaker induction of the promoter was evident approximately 3-6 mm away from the lesion. As well, activity was also observed in portions of the leaf which had not been infected. However, no significant β-glucuronidase activity was apparent in leaves above or below the leaf that had been inoculated with the fungal spore suspension.

Broglie and co-workers (1989; 1993) also investigated where the fungal mycelia were concentrated within the leaf. Although fungal mycelia were found to be concentrated within the necrotic lesion significantly fewer were found at the periphery of the lesion in the region that exhibited GUS enzymatic activity. Basically, no fungal structures were apparent beyond this zone. These findings are very interesting as while the distribution of the fungus was similar to the distribution of GUS activity the activation of the bean chitinase promoter was present in regions that had not been exposed to the fungus. Similar results have been observed with tobacco plants infected with *R. solani* and *Sclerotium rolfsii*. From these findings it is evident that the activation of the bean chitinase 5B promoter is dependent on the presence of the fungus as well as the colonization and damage to the plant tissue (Roby *et al.*, 1990; Broglie *et al.*, 1993).

The mechanism responsible for induction of the bean chitinase gene expression has been studied using a transient gene expression system in bean protoplasts (Roby *et al.*, 1991). The advantage of employing such a system is that protoplasts allow for the study of transcriptional regulation quickly and efficiently by avoiding the potential complications of position-effect and variable gene copy number. By creating 5′ deletions in the 5B chitinase promoter-GUS construct (Broglie *et al.*, 1989; Roby *et al.*, 1991) it

was determined that upon deleting the promoter to -453 a decrease in GUS activity of 38% occurs in the presence of 1-aminocyclopropane-1-carboxylic acid, the direct precursor of ethylene. Roby and co-workers (1991) also found that further shortening of the promoter segment to -305 resulted in a 62% decrease in GUS activity. Furthermore, upon the removal of sequences between -305 and -236 the enzymatic activity was observed to decline to the control levels displayed in extracts of untreated protoplasts. Therefore, the DNA sequences between -305 and -236 are essential for induction of the reporter gene by ethylene and elicitors.

While the promoter of the bean chitinase gene was being studied, the expression of the *Arabidopsis* acidic chitinase promoter and its response to fungal pathogens were also being investigated (Samac and Shah, 1991). In this study a chimeric gene composed of 1129-bp of the 5′ upstream sequence from the chitinase gene was fused to the GUS coding region and used to transform tomato and *Arabidopsis* plants. Using histochemical and quantitative assays it was revealed that in transgenic tomato plants GUS expression was induced around necrotic lesions caused by *Phytophthora infestans* and *Alternaria solani* (Samac and Shah, 1991). Further studies undertaken using 5′ deletions confirmed that the proximal 192-bp from the transcription initiation site was sufficient to establish constitutive and induced patterns of expression. Specifically, a negative regulatory element was found between -384 and -590 bp and positive regulatory elements between -1129 and -590 bp.

Chitinase promoters isolated from tobacco, rice and peanut have also been studied. The promoter of the tobacco Class I endochitinase gene *CHN50* has also been found to be regulated by pathogen infection, elicitor, salicylate, ethylene and mechanical

wounding (Fukuda *et al.*, 1991; van Buuren *et al.*, 1992). Within this promoter an elicitor-response element (EIRE) was identified that is able to drive the expression of GUS in transgenic tobacco calli in response to elicitors from *Phytophthora infestans* cultures (Fukuda and Shinshi, 1994). Wounding and elicitor treatment of tobacco plants carrying a 1.5-kb promoter-GUS fusion from the rice chitinase gene *RCH10* also resulted in GUS expression (Zhu *et al.* 1993). However, in this case ethylene did not appear to have any effect on the expression of this gene. Reverse transcriptase PCR has also been used to demonstrate that peanut carries chitinase genes that are tightly regulated by treatment with cell wall components from *Phytophthora megasperma.* Expression studies carried out with *A.h.Chi2;1* promoter deletions indicated that pathogen induction was due to a region located between positions -431 to -441 bp. The sequence within this area, TCAGAAAGTCA, displays the core sequence of the EIRE characterized from the tobacco gene (Kellmann *et al.*, 1996). These findings suggest that this element is probably very important is controlling the expression of chitinase genes in plants.

Leah and co-workers (1991; 1994) have characterized a chitinase protein from barley seeds that displays an aleurone-specific pattern of expression. The gene, *Chi26,* encoding this protein has been studied at the molecular level to determine what *cis*-acting DNA sequences are responsible for controlling the tissue specific expression. Analysis of *Chi26* 5' and 3' promoter deletions fused to the GUS gene has indicated that sequences between -200 and -140 bp confer developmental and aleurone-specific expression. By using deletions and replacements it has been found that sequences specifically between -179 and -147 bp direct the expression of chitinase in aleurone cells (Leah *et al.*, 1994). Examination of this 33-bp region has determined that it activates transcription

specifically in aleurone. These results indicate that there are *cis* elements present in this chitinase promoter that specifically direct expression in certain plant tissues.

3.1.8.5 Subcellular-localization of plant chitinases

To learn more about the possible role chitinase has in plants it is necessary to determine where the enzyme accumulates in stressed tissue. With a few exceptions, where chitinases have been reported to accumulate in the vacuole, most chitinases have been reported to be located apoplastically. Groups studying the location of chitinases in plants based their findings on the presence of the enzyme in intercellular washing fluids collected from infected leaves (Boller and Vogeli, 1984; Legrand *et al.*, 1987; Kombrink *et al.*, 1988). There are, however, problems involved with the collection of intercellular washing fluids from infected tissue. Since many pathogen-induced proteins accumulate at very high levels around the sites of infection, lysis of these cells during the process most likely alters the protein pattern of the intercellular washing fluids. Therefore, using this technique it is impossible to determine whether or not the chitinases are actively secreted or released into the extracellular space due to host cell lysis. To overcome this problem immunocytochemical and cell fractionation studies have been used by several research groups to localize different chitinase isozymes (Boller and Metraux, 1988; Fink *et al.*, 1988; Flemming *et al.*, 1991; Mauch and Staehelin, 1989; Wubben *et al.*, 1992). The general conclusion from such studies is that there are chitinases that accumulate in both places and that those chitinases which accumulate in the vacuole belong to Class I.

Mauch and Staehelin (1989) studied the subcellular localization of a Class I bean chitinase using immunogold cytochemistry and biochemical fractionation techniques. The

resulting micrographs clearly indicated that this enzyme accumulates in the vacuole of ethylene-treated leaves. Biochemical fractionation experiments further confirmed this result as chitinase was not present in intercellular washing fluids collected from the treated leaves.

Studies have demonstrated that a C-terminal extension of approximately 6 amino acids is required for vacuolar localization of the basic Class I tobacco chitinase (Neuhaus *et al.*, 1991). Such sequences are present in basic vacuolar chitinases of bean, potato, *Arabidopsis* and poplar (Broglie *et al.*, 1986; Gaynor, 1988; Parsons *et al.*, 1989; Samac *et al.*, 1990; Shinshi *et al.*, 1990) thus suggesting that this short sequence is important for vacuolar targeting. Class IV chitinases produced by canola and sugar beet, as well as certain Class I chitinases such as those from pea, rice and barley, lack this short sequence (Huang *et al.*, 1991; Mikkelsen *et al.*, 1992; Rasmussen *et al.*, 1992; Vad, 1991). This suggests that these particular chitinase enzymes are located extracellularly. The Class IV sugar beet chitinase has been confirmed as being present in the apoplast (Mikkelsen *et al.*, 1992).

3.1.8.6 Possible roles of chitinases in plants

One question concerning the location of chitinase in the vacuole of plant cells is at what time does this enzyme intervene in pathogenesis. Since several chitinases are not located in the cell wall it has been proposed that this enzyme may only intervene at late stages of pathogen attack as in a hypersensitive or necrotrophic reaction. However, it is possible that the different isozymes of chitinases present in the plant may possess different roles.

It has been well documented by several *in vitro* studies that a growth inhibitory effect against fungi containing chitin in their cell walls is observed in the presence of chitinase (Schlumbaum *et al.*, 1986; Roberts and Selitrennikoff, 1988; Broekaert *et al.*, 1988). Examination of the apex of the growing fungal hyphae has shown that nascent chitin chains are produced that are accessible to hydrolysis by chitinase. In the mature cell walls distant from the apex the chitin-glucan fibres are overlaid with protein and polysaccharide layers and, therefore, it is not as easy for chitinase to attack these regions. A destructive effect of chitinase treatment on immature fungal cell walls has been observed by several research groups (Broekaert *et al.*, 1988, Ordentlich *et al.*, 1988). However, Mauch and co-workers (1988) found that pea chitinase alone could not inhibit the growth of most of the fungi tested.

From the various studies carried out a possible model for the role of chitinase in the plant defense response has been proposed by Mauch and Staehelin (1989): when a fungal pathogen penetrates the plant surface it proceeds into the intercellular space of the host. At this point the hyphae come into contact with another defense protein known as β-1,3-glucanase which has been shown to accumulate in the intercellular spaces. It has been hypothesized that upon contact the β-1,3-glucanase acts to release oligosaccharide fragments from the β-1,3-glucan containing fungal cell wall. These oligosaccharides in turn act as elicitors. The elicitors induce several of the defense genes including those encoding chitinases. Chitinases would thus act as a last line of defense as they are released when the attacked host cell lyses. There are several advantages in this mode of action. First, in the early stages of infection the concentration of the chitinase enzymes may be too low to interfere adequately with the growth of the hyphae. By accumulating

in the vacuole the release can be delayed until there is an effective level of the enzyme available. In this case, the sudden release of the enzyme would most likely prevent the fungus from becoming adapted to the changing conditions.

There is now direct evidence that chitinase interacts with the invading fungal cell wall as immunocytochemical techniques have shown that the enzyme accumulates around the fungal cell walls (Wubben *et al.*, 1992). Furthermore, it has been suggested that in those plants where chitinases also accumulate extracellularly they are involved in the induced response. Thus they most likely work with the β-1,3-glucanases to release oligosaccharides that can act as elicitors.

Although a lot of research has focused on the role of chitinases in a plant defense against invading pathogens it should be noted that chitinases appear to be involved in some other plant functions. De Jong and co-workers (1992) have demonstrated that an acidic endochitinase plays an important function in early somatic embryo development in carrot. In this system chitinase was found to allow completion of embryo development in a temperature-sensitive carrot mutant cell line at non-permissive temperatures. In search of determining a possible function for the chitinase enzyme in plant embryogenesis, De Jong and co-workers (1993) tested several compounds that contain oligomers of N-acetylglucosamine for their ability to promote embryo formation. What is interesting is that of all the different compounds tested only the *Rhizobium* lipo-oligosaccharides or Nod factors were found to be effective in rescuing the formation of temperature sensitive embryos. From the results obtained by this research group it seems that N-acetylglucosamine-containing lipo-oligosaccharides of bacterial origin can mimic the effect of the carrot endochitinase. Therefore, it is possible that this chitinase may be

involved in the generation of plant analogs of the *Rhizobium* Nod factors. In other words, it could be that the endochitinase releases a signal molecule that is analogous to the Nod factors from a yet unidentified N-acetylglucosamine-containing precursor that is present in low concentrations in the cell wall. As well, chitinases may participate in the generation of signal molecules that regulate plant development such as organogenesis. It is evident that to further define these possible roles for plant chitinases it is necessary to search for endogenous chitinase substrates within in the plant (Collinge *et al.*, 1993).

3.1.8.7 Expression of foreign chitinase genes in plants

The transfer of foreign chitinase genes into plants is not a very recent concept. A number of groups have introduced and secured the expression of appropriate cloned chitinase sequences for the purpose of, hopefully, improving crop protection and ultimately production. One of the first groups to transfer successfully a foreign chitinase gene to plants used the *chi*A gene of *Serratia marcescens* (Taylor *et al.*, 1987). This group fused the coding region of the *chi*A gene to the promoter and 3′ polyadenylation region of the *A. tumefaciens* nopaline synthase gene and the resulting construct was transferred to tobacco. Examination of transgenic plants carrying the gene, in which specific nucleotides around the initiating AUG codon were altered, revealed an eight-fold increase in the amount of chitinase protein present. Upon analyzing the *chi*A mRNA it was found that the level of mRNA also increased. From the analysis of these plants it was found that approximately 50% of the chitinase protein produced in the transformed tobacco plants had the same molecular weight as the protein secreted from *S. marsescens* (Dunsmuir and Suslow, 1989). In order to obtain high-level expressions of the ChiA

protein the gene was fused to two different chlorophyll a/b binding gene promoters from petunia, which allow for leaf-specific expression, to the CaMV 35S promoter and to a promoter that directs expression to tobacco roots (Jones *et al.*, 1988; Franck *et al.*, 1980). In each case, the expression of the gene in the roots and leaves paralleled that observed in the callus tissue which was transformed with *chi*A fused to the *nos* (nopaline synthase) promoter. In those transformants that showed the highest levels of *chi*A expression it was found that approximately 0.25% of the total soluble leaf protein was ChiA.

Homozygous tobacco lines with a single *chi*A locus have been used to evaluate whether or not a increase in resistance to chitin-containing pathogens occurs (Dunsmuir and Suslow, 1989). The chitinase levels were measured in *chi*A-transformed plants and control plants that contained the T-DNA binary vector, but not the *chi*A gene. The results from these experiments demonstrated that there is no significant difference between the level of chitinase activity detected in untransformed tobacco and those plants transformed with the control vector. However, in transformed plants carrying *chi*A the increase in chitinase activity was approximately 25% above the endogenous levels. These plants were assessed for their susceptibility to infection by the tobacco brown-spot pathogen *Alternaria longipes*. By using a leaf disc assay it was shown that there is a significant reduction in the mean lesion diameter and severity of disease in the transgenic plants.

The chitinase sequence of *Phaseolus vulgaris* has also been transferred to tobacco (Broglie *et al.*, 1989). Transformed plants have been assayed for chitinase activity and it has been shown that when transformed plants are grown in air little or no expression of the bean chitinase genes occurred. However, upon exposure to an atmosphere containing 50 parts per million ethylene, the level of bean chitinase mRNA

increased 20-fold to 50-fold indicating that the expression of this gene is modulated by exogenous ethylene.

Transgenic tobacco and canola seedlings constitutively expressing the bean chitinase gene under the control of the CaMV 35S promoter have been examined (Broglie *et al.*, 1991). Assays done on protein extracts from transformed tobacco plants indicated an increase in chitinase activity. As well, homozygous progeny showed a 2- to 4-fold increase in the roots and a 23- to 44-fold increase in the leaves over the levels of chitinase produced in the control plants. These results indicate that the chimeric 35S-chitinase gene gives constitutive expression of the bean polypeptide in healthy plants.

The tobacco plants were also assayed for resistance to the phytopathogen *Rhizoctonia solani.* It has been well established that when seeds are planted in soil heavily infested with *R. solani*, seedlings have problems with early season growth and stand establishment. To determine susceptibility to fungal attack 18-day old homozygous progeny of the transgenic tobacco plants were grown in the presence of *R. solani* (Broglie *et al.*, 1991). Transgenic plants which produced higher amounts of the bean polypeptide clearly showed greater resistance to the development of disease symptoms. When such plants were grown in the presence of pathogens that lacked a chitin in their cell wall no difference in survival was detected when compared to control plants. Although the extent of resistance observed in the 35S-chitinase plants varied with the amount of fungal inoculum applied, it was found that a delay in the appearance of symptoms occurred as well as lower severity of the disease.

Transgenic canola plants containing high constitutive levels of bean chitinase have also been used to study the cytochemical aspects of chitin breakdown *in vivo*

(Benhamou *et al.*, 1993). Analysis of root tissues of infected wild-type canola plants that had been infected with *R. solani* has revealed that this fungus is capable of extensive tissue colonization including the xylem. As the pathogen ingressed towards the vascular system it was found that marked host cell wall alterations occurred in advance of fungal penetration. When the transgenic canola plants were examined it was found that the pattern of fungal colonization was different from that observed with the wild-type plants. For example, even though penetration of the host cuticle and epidermis was observed, fungal colonization was usually restricted to the cortex. In every case examined it was found that severe hyphal alterations ranging from increased vacuolization to cell lysis occurred. Results from several cytochemical studies indicated that hyphal alterations correlated with extensive chitin degradation. Therefore, Benhamou *et al.* (1993) concluded that the reduction in fungal biomass and the increase in hyphal alterations are typical features that occur in plants that express the bean chitinase gene.

The introduction of foreign chitinase genes into plants has become very common in the past couple of years. For example, a drought-induced chitinase gene has been isolated from a wild relative of cultivated tomato found in South America known as *Lycopersicon chilense* (Agharbaoui *et al.*, 1996). This gene has been transferred to 5 genotypes of cultivated tomato using the CaMV 35S promoter. Some of the transgenic plants were tested for resistance to *Verticillium dahliae* and they were found to have enhanced resistance when measured bys foliar disease symptoms, leaf disease index, and vascular discolouration index. A report has recently been published on the transfer of chitinase genes isolated from petunia, tobacco and bean to carrot and cucumber plants (Punja *et al.*, 1996). Transgenic plants carrying these different genes have been tested

against several different plant pathogens. In studies using whole plants and *in vitro* inoculations with detached leaves it was found that there was no difference in disease development between transgenic and nontransgenic cucumber plants when inoculated with *Alternaria cucumerina, Botrytis cinerea, Colletotrichum lagenarium* and *R. solani.* However, in the case of the carrot plants it was found that the extent of lesion development was lower upon inoculation with *B. cinerea, R. solani* and *S. rolfsii,* in the transgenic plants expressing the basic tobacco gene, but not in those plants expressing the acidic petunia chitinase gene. As well, there were no detectable differences with *Alternaria readicini* or *Thielaviopsis basicola.*

3.1.9 The *chiB* gene of *Serratia liquefaciens*

The set of genes that determine the expression of enzymes involved in chitin degradation by *S. liquefaciens* were originally isolated by Joshi *et al.* (1988). From this research it was found that the set of genes included one chitinase gene (*chiB*), one chitobiase gene (*chiC*) and a region referred to as *chiD* whose absence led to higher expressions of the *chiB* and *chiC* genes. Further molecular genetic characterization of the chitinase regulon of *S. liquefaciens* has revealed that *chiB* is 1499-bp in length and gives rise to a polypeptide with a molecular weight of approximately 52-kDa that displays strong chitinase activity (Woytowich, 1991). Preliminary findings have also suggested that the expression of *chiB* is controlled by an RNA folding mechanism.

Analysis of the amino acid sequence of the chitinase polypeptide of *S. liquefaciens* has revealed that it is 93.6% identical to the ChiB polypeptide of *S. marcescens* (Woytowich, 1991). Both of these polypeptides consist of 499 amino acids

and the homology between the two begins at the first amino acid and extends to amino acid 450. A small portion of the *S. liquefaciens* ChiB polypeptide was also found to share homology to the ChiA polypeptide of *S. marcescens*. This homology, however, is restricted to an overlap of 81 amino acids and that within this region there is 32.1% homology.

From the research undertaken by Woytowich (1991) it is evident that the *chiB* gene of *S. liquefaciens* does not share any significant homology to other bacterial or plant chitinase genes that have been transferred to plants. For this reason, and because the transfer of other chitinase genes of bacterial and plant origin have led to the regeneration of plants with increased disease resistance, it was of interest to know whether or not transgenic plants carrying *chiB* displayed similar or even better disease resistance characteristics.

3.2 Results

3.2.1 Genetic engineering of the *chiB* gene of *S. liquefaciens*

Since most prokaryotic genetic control elements do not function in plants, it is necessary for alternative controlling elements that operate in plants to be present in order for a bacterial gene to be expressed in the plant. The minimum controlling elements required are a promoter and a terminator. In this study the *chiB* gene of *S. liquefaciens* was transferred to the tobacco genome under the control of a partially duplicated 35S cauliflower mosaic virus (CaMV) promoter and a nopaline synthase (*nos*) terminator. A partially duplicated 35S CaMV promoter was used because it is known to enhance transcription (Kay *et al.*, 1987).

In order for the *chiB* gene to be cloned into a plant vector for subsequent transformations it first had to be cloned into a plant cassette which contains the CaMV 35S-35S promoter and *nos* terminator. For this cloning to be successful it was necessary to introduce two restriction endonucleases, a *Nco* I site at the initiation codon and a *Bgl* II site after the termination codon, by site-directed mutagenesis (SDM). By introducing this site the sequence of the 5′ end of the gene had to be slightly altered by the addition of a coding sequence for an amino acid so that the ORF of *chiB* remained in the correct frame. The *Nco* I and *Bgl* II sites were introduced by SDM using the plasmid pAW379. A summary of the different plasmids constructed during this study is given in Appendix A.

To ensure that the introduction of the additional amino acid did not affect the chitinase activity of the altered *chiB* gene, it was cloned into the expression vector pSE380 and assayed for chitinase activity using the radiochemical assay of Molano *et al.*

(1977). As illustrated in Table 3.2 the altered gene still produced a protein with chitinase-like activity.

The *Nco* I - *Bgl* II fragment containing the *chiB* ORF was cloned between the *Nco* I and *Bam* HI sites of the plasmid pBI524-14. (R.S.S. Datla PBI-NRC, Saskatoon, unpublished). The plasmid pBI524-14 contains the partially duplicated 35S CaMV promoter, the alfalfa mosaic virus coding region and a polyadenylation terminator region (*nos* terminator). In this study the 5′ and 3′ ends of the *chiB* gene were sequenced and the resulting plasmid was designated pAW444. Protein extracts were isolated from the *E. coli* DH5α strain carrying this construct and assayed to determine if chitinase activity could be demonstrated under the control of the 35S-35S CaMV promoter. As shown in Table 3.3 the construct pAW444 does express *chiB*.

In this study, the "35S-35S + *chiB* ORF + *nos*" expression cassette was dropped out of pAW444 as a *Xba* I - *Xba* I fragment and ligated into the plant vector pRD400 (Datla *et al.*, 1991) to obtain pAW445 (Fig.3.1). The plasmid pRD400 is a binary plant transformation vector carrying an *npt II* gene for genetic selection in plants. The correct orientation of the expression cassette was determined by restriction endonuclease digests and sequencing. This construct was also tested for the production of chitinase and as illustrated in Table 3.3 it was positive for chitinase activity. This plasmid is capable of replication and maintenance in *A. tumefaciens* and the transfer DNA region is illustrated in Fig. 3.2.

Table 3.2 Chitinase activity in bacterial protein extracts[1]

Reaction	Source of Protein Extract	^{3}H chitin solubilized per reaction (cpm) 30 μg protein	50 μg protein
1.	Control- DH5α	213	210
2.	Boiled DH5α [pAW439]	210	115
3.	DH5α[pAW439]	5197	4761
4.	Boiled DH5α [pAW440]	149	74
5.	DH5α [pAW440]	6622	4228

[1.] The chitinase assay of Molano *et al.* (1977) was used to determine the ^{3}H counts released into the soluble fraction from the insoluble tritiated chitin used as the substrate. Each of the values is the average of the results obtained in two separate experiments.

Table 3.3 Chitinase activity in bacterial protein extracts

Reaction	Protein Extract	^{3}H chitin solubilized per reaction (cpm)[1] 25 µg protein/reaction
1.	Control- DH5α	277
2.	DH5α [pAW444]	2076
3.	DH5α [pAW445]	1056

[1.] Each of the values is the average of the results obtained in two separate experiments.

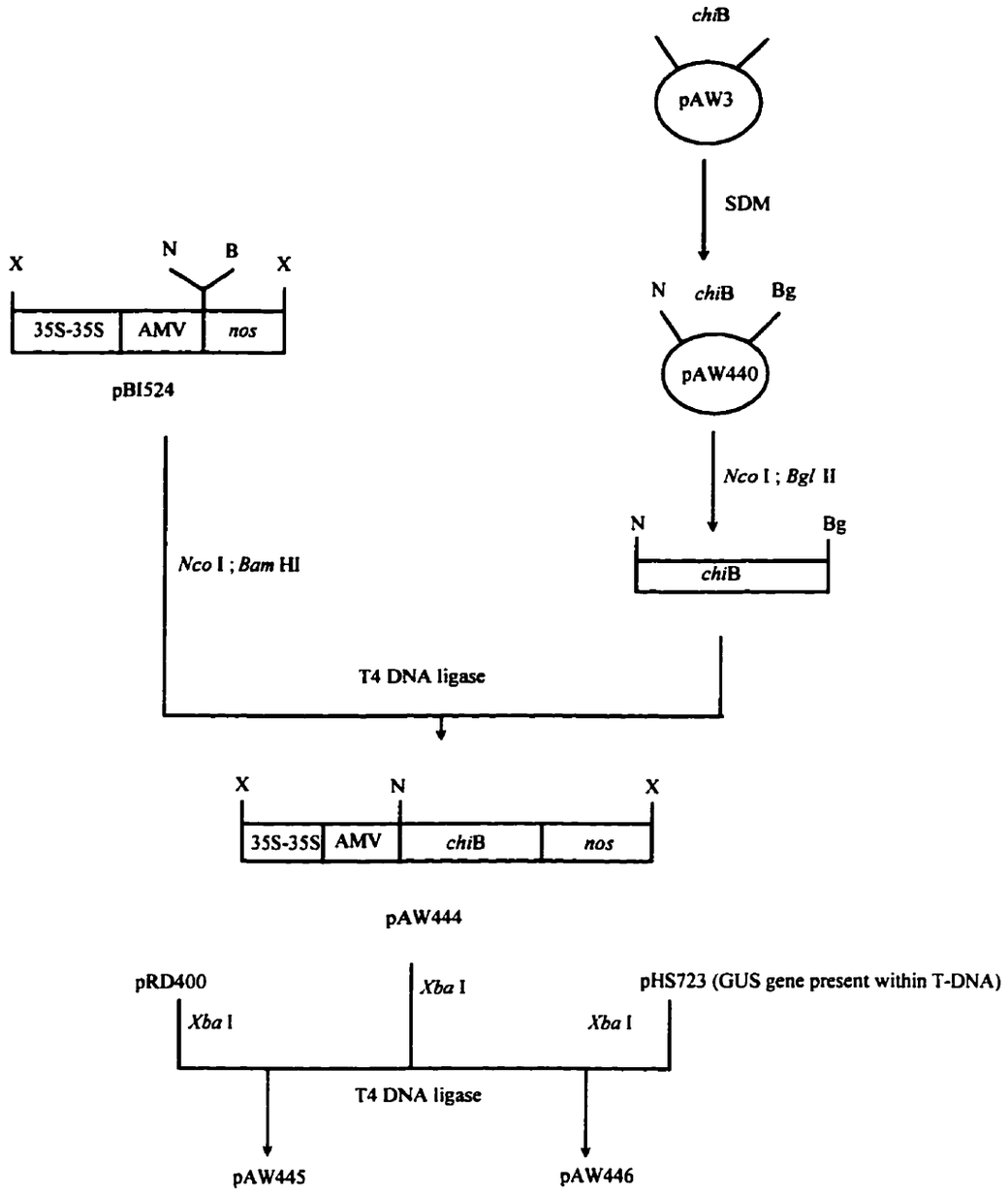

Figure 3.1. Construction of the *chi*B expression cassette and vectors. 35S-35S, partially duplicated CaMV 35S promoter; AMV, alfalfa mosaic virus enhancer; *nos*, nopaline synthase terminator; *chi*B, open reading frame of *S. liquefaciens* chitinase B gene; SDM, site-directed mutagenesis. Key restriction sites are abbreviated as B, *Bam* HI; Bg, *Bgl* II; N, *Nco* I; X, *Xba* I.

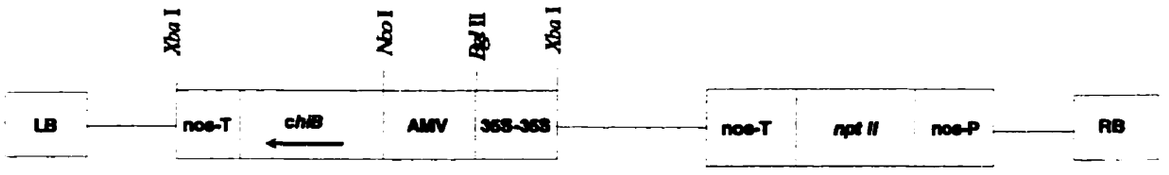

Figure 3.2.Transfer DNA region of the plasmid pAW445 used for tobacco and canola transfer. LB and RB, left and right border of the T-DNA; nos-P, nopaline synthase gene promoter; nos-T, nopaline synthase gene terminator; *npt* II, neomycin phospho-transferase gene. See Figure 3.1 for other abbreviations

3.2.2 Transformation of tobacco with the engineered *chiB* sequence

Agrobacterium tumefaciens has the capability of transferring DNA to plant cells and the DNA transferred is commonly known as the T-DNA (Zambryski, 1992). The *A. tumefaciens* strain GV3101 (Koncz and Schell, 1986) carries the plasmid pMP90 which is a deletion derivative of the Ti plasmid that has lost the T-DNA. This plasmid provides the virulence functions that are required to transfer the T-DNA from the bacterial plasmid to the plant cells. In this study the plasmid pAW445, which carries the *chiB* expression cassette in its T-DNA region, was transferred into the *A. tumefaciens* GV3101 strain by the freeze-thaw transformation method (Holsters *et al.*, 1978). The integrity of this plasmid was confirmed by restriction enzyme digests and PCR. Restriction digestion of pAW445 with the enzymes *Xba* I and *Hind* III resulted in DNA fragments of approximately 12-kb, 2.5-kb and 14-kb, 0.6-kb, respectively. DNA fragments of these sizes were observed when plasmid DNA was extracted from *E. coli* DH5α[pAW445] and *A. tumefaciens* GV3101[pAW445] and digested with the same enzymes. These results indicated that pAW445 was present and stable in *A. tumefaciens* GV3101. The presence of *chiB* in the *A. tumefaciens* strain was further confirmed by PCR. Two sets of oligonucleotides were used to amplify the entire 1.5-kb *chiB* gene and a 600-bp portion of the gene by PCR. In reactions containing plasmid DNA extracted from both the *E. coli* and *A. tumefaciens* strains bands of the expected size were amplified.

Tobacco leaf discs were co-cultivated with the *A. tumefaciens* strain carrying pAW445 and transformants were selected for growth on MS media containing the antibiotic kanamycin. Kanamycin can be used for selection as the transformed cells carrying the T-DNA are capable of inactivating the antibiotic by means of

phosphorylation by the product of the *nptII* gene. The transformation frequency of tobacco is generally quite high (at least 80%), but when the first transformation was done using pAW445 a very low apparent transformation frequency of approximately 15% was observed as only 15 tobacco plants were regenerated from 100 infected discs.

3.2.3 Identification of *chiB* in tobacco

To test for the presence of *chiB*, PCR was carried out on DNA extracted from the 15 tobacco plants as well as from wild type plants using the two sets of oligonucleotides which amplify either the entire *chiB* gene or only a portion of it. In this experiment, however, no DNA fragments were amplified from any of the 15 putative *chiB* plants. To ensure that the oligonucleotides being used for this experiment did indeed amplify the *chiB* gene PCR was carried out on plasmid DNA that had been extracted from the strains DH5α[pAW445] and GV3101[pAW445]. In each reaction run DNA fragments of the expected size were amplified.

Since PCR did not confirm the presence of *chiB*, a non-radioactive Southern blot analysis was conducted. Genomic DNA extracted from wild type tobacco plants and the 15 regenerated plants was digested with the enzymes *Xba* I and *Bam* HI, immobilized onto nylon membranes and probed with the labelled *chiB* ORF. As a positive control plasmid DNA isolated from the strain DH5α[pAW445] and digested with *Xba* I or *Bam* HI was also immobilized onto the membranes. If *chiB* is present in the genome, the restriction enzyme *Xba* I should cut at two sites and drop out the *chiB* expression cassette as a 2.4-kb fragment and *Bam* HI should cut at two sites within the *chiB* ORF and drop out a 800-bp fragment of *chiB*. One would then expect bands of these sizes to be present

when probed with the *chiB* ORF. As well, depending on the number of T-DNA's present in the genome other fragments of variable sizes may also be present. Unfortunately, when the membranes were probed only the control bands appeared. The non-radioactive Southerns were repeated using a new detection kit and different portions of the *chiB* ORF, but in all cases the results were negative for the presence of *chiB*. The Southerns were repeated using a radiolabelled probe and different stringency conditions but no bands appeared indicating that the *chiB* sequence was absent and all of the plants regenerated were escapees. These plants, therefore, were excluded from the study.

The tobacco transformation using pAW445 was repeated, but no tobacco plants were regenerated as all of the 200 infected discs turned yellow and failed to form calli. A third transformation was done involving approximately 300 discs and once again the same results were obtained. From these transformations it appeared as if the T-DNA was not being transferred to the plant cells as control discs that had not been infected, but were placed on selection plates, turned yellow and failed to produce calli.

While performing the tobacco transformations *B. napus* transformations were also undertaken. Due to the normally low regeneration frequency of this transformation (5%) over 2000 cotyledons were infected with the *A. tumefaciens* strain GV3101[pAW445]. However, from this transformation no canola plants were regenerated. A second and third transformation was performed using 3000 and 5000 cotyledons, respectively. Once again no plants could be regenerated. Examination of the infected cotyledons revealed that as in the tobacco transformations calli were not forming around the infected tissue of the cotyledon, but rather the tissue was turning yellow and dying.

3.2.4 Cloning the *chiB* expression cassette into pHS723

While struggling with the pAW445 tobacco and *B. napus* transformations a new plant vector, pHS723 (G. Selvaraj, PBI/NRC, Saskatoon, unpublished), became available. The advantage of pHS723 over pRD400 is that it has a GUS gene present on it which allows for the quick screening of a potential transgenic plant by a leaf disc GUS assay. The *chiB* gene was then cloned into pHS723 as previously described for pRD400. The expression cassette was dropped out of pAW444 as a *Xba* I fragment and cloned into the *Xba* I site of pHS723. The presence of *chiB* in pHS723 was confirmed by restriction enzyme digests and PCR. *Agrobacterium tumefaciens* GV3101 was transformed with this construct, designated pAW446, and its presence also confirmed by restriction enzyme digests and PCR.

3.2.5 Transformation of tobacco with pAW446 and identification of putative transgenic plants

Due to the low regeneration frequency normally observed with *B. napus* transformations it was decided that the *chiB* research using pAW446 would focus on tobacco transformations. The *A. tumefaciens* GV3101 strain carrying pAW446 was used to infect 200 sterile tobacco leaf discs. Two weeks after the discs were infected with GV3101[pAW446], a majority of these started to yellow and calli were never formed. To ensure that the time allowed for co-cultivation was sufficent 100 of the infected discs were allowed to remain on the co-cultivation media for 7 days. Although heavy *Agrobacterium* growth was apparent around the discs, they were transferred to selection

media and approximately six weeks after this transfer calli appeared to form around some of the discs (Fig. 3.3). A total of 12 plants were regenerated from 100 discs and GUS assays were undertaken on the leaves from those shoots being transferred to rooting media. GUS assays undertaken on these 12 plants indicated that seven of them were positive for GUS activity. GUS activity was monitored in the 12 plants as they formed roots and were transferred to potting soil. Most of the plants that were determined to be GUS negative did not root and were considered to be escapees. As the GUS positive plants grew it was found that the GUS activity present in the leaves continued to decrease until all of the originally GUS positive plants tested negative. These plants were tested for the presence of *chiB* by PCR and non-radioactive and radioactive Southerns and in each case *chiB* could not be detected. These results demonstrated that false GUS results occurred possibly because of the bacteria still present in the system when tested which indicates that the determination of positive transgenic plants should not be based solely on the initial results of the GUS assay.

Tobacco leaf discs were infected with pAW446 for a second time and although the majority of the 200 infected discs yellowed, calli did form on some. In this transformation, designated pAW446a, 32 shoots developed. Most of these shoots were pale-, rather than a lush-green, and when the leaves were tested for GUS activity 22 out of the 32 were positive. Examination of the GUS positive leaves under a stereomicroscope revealed that the majority of the GUS activity was on the surface or hairs of the leaves. This type of reaction suggests that the majority of positive reactions were probably due to the presence of residual *A. tumefaciens*. Crude protein extracts were

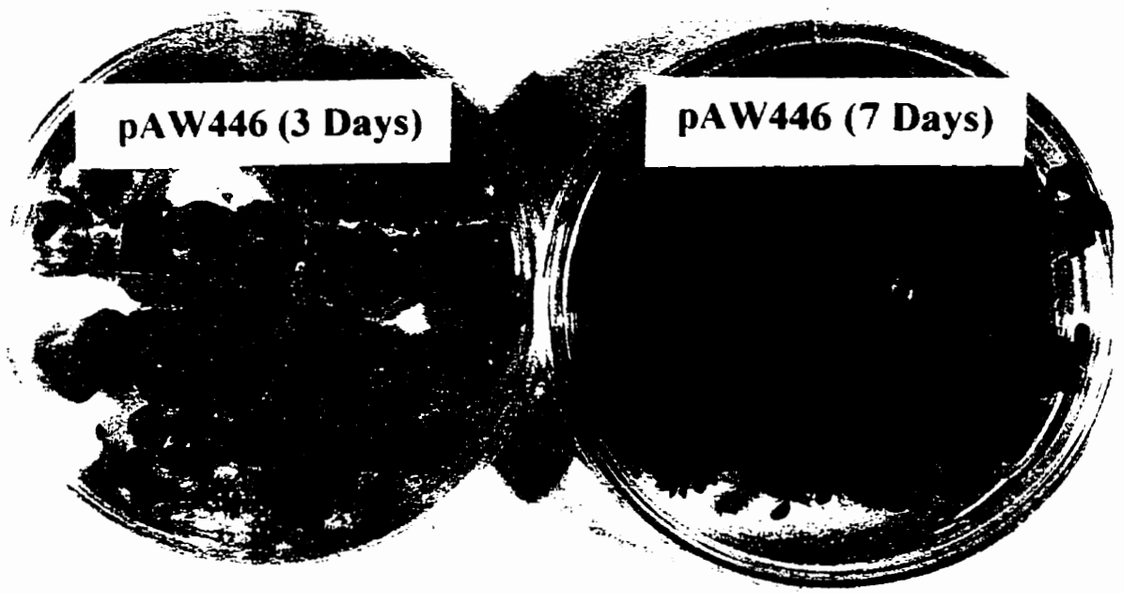

Figure 3.3. A comparison of tobacco discs infected with *A. tumefaciens* GV3101[pAW446], incubated on co-cultivation media for three or seven days , and then transferred to selection and shooting media for two weeks and four weeks, respectively.

isolated from the leaves of plants that had been in potting soil for approximately 12 weeks and continued to show GUS activity. Chitinase assays were performed to determine if any of the potentially *chiB* positive plants produced significantly high levels of chitinase. As can be seen in Table 3.4, the results obtained in this set of experiments were inconclusive as some of the numbers were quite variable. Although different concentrations of protein were tested from the different plants only the GUS positive plants pAW446a-15 and pAW446a-18 showed an increase in chitinase production. Radioactive Southerns were performed on DNA extracted from pAW446a-15, pAW446a-18 as well as the other plants that were GUS positive. Two different radioactive probes, the *chiB* ORF and the *nptII* gene, were used to probe the membranes. No bands appeared on any of the Southerns indicating that *chiB* had not integrated into the genome of these plants. These results were further confirmed by PCR. It appears as if the GUS positive results and the slight increase in chitinase levels observed in some of the strongly GUS positive plants were due to residual bacteria.

Due to the high number of false GUS positive plants observed in this study an experiment was undertaken to compare the observation of GUS activity with the number of *A. tumefaciens* cells. In this experiment serial dilutions of a culture containing $1.5x10^8$ cfu of *A. tumefaciens* GV3101[pHS723] were prepared and incubated in the presence of 200 μL of X-Gluc for 24 hours at 28°C. When the cells were spun down and visualized under a stereomicroscope it was found that GUS activity could be observed up to the presence of $1x10^4$ cfu. However, once the culture had been diluted to $1x10^3$ cfu no GUS activity could be observed under the stereomicroscope. These results clearly show

Table 3.4 Chitinase assays done on proteins extracted from GUS positive plants

Reaction	Plant Extract	^{3}H chitin cpm solubilized per protein reaction [1]			
		25µg protein	+/- s.e.	100µg protein	+/- s.e.
1.	wild type	291	32	597	33
2.	pAW446a-10	804	44	810	55
3.	pAW446a-13	769	67	317	22
4.	pAW446a-14	455	41	355	21
5.	pAW446a-15	715	87	1116	110
6.	pAW446a-18	536	33	1097	36
7.	pAW446a-27	235	11	411	22
8.	pAW446a-31	442	15	468	17
9.	control (no protein present)	292	21	-	-

[1] The chitinase assay of Molano *et al.* (1977) was used to determine the ^{3}H counts released into the soluble fraction from the insoluble tritiated chitin used as the substrate. The values are the average results of three separate experiments.

that GUS activity can be observed in the presence of very low numbers of bacterial cells, therefore it is not necessary to have high numbers of residual bacterial in order to have a false positive result.

A third and fourth transformation were carried out and again in each case the 200 infected discs yellowed and failed to form calli. To ensure that the plasmid pAW446 was present in the *A. tumefaciens* strain being used to infect the leaf discs, DNA was extracted from the bacterial cultures growing around the discs present on the co-cultivation plates. PCR was undertaken using the DNA extracted from the bacteria and in every case a fragment of the expected size for *chiB* was amplified thus confirming that the bacteria used in the transformations likely harboured the plasmid pAW446. At this point it was decided that the transformation procedures and the components used for each transformation should be examined to determine if a specific lot number of one of the components was inhibiting the transformation process thus preventing callus formation. As illustrated in Table 3.5, the lack of transformation and the formation of calli and shoots is not due to one specific component as the calli and shoots formed for each transformation undertaken using the vector pHS723 worked each time, but failed to form when the construct pAW446 was used. Control transformations with pHS723 ruled out a problem with the vector itself as a large number of transgenic tobacco and canola plants were regenerated, and confirmed to contain pHS723 by PCR and TA cloning, from a single transformation . To determine if the concentration of the kanamycin being used for selection was too high, a separate transformation involving 200 discs was undertaken

Table 3.5 Different components tested to determine if any had a negative effect on the formation of calli and shoots of infected tobacco discs

Component tested	Results of Transformation[1]	
	pHS723	pAW446
None	+	-
2,4-D	+	-
BA	+	-
NAA	+	-
MS media lot number	+	-
Phytagar	+	-
Sucrose	+	-
Kanamycin	+	-
Carbenicillin	+	-

[1] Results indicate success (+) or failure (-) of the formation of calli and shoots.

using 80% of the discs infected with GV3101[pAW446] and placed on the selection plates containing 50 μg/mL of kanamycin formed calli and shoots. GUS assays done on the seedlings that had been transferred to rooting media indicated that 34% of them were escapees. PCR and Southern analysis on DNA extracted from these plants indicated, however, that the *chiB* gene had not integrated into the genome of the plants.

The fact that in this transformation more escapees were regenerated than in any of the other transformations is to be expected as the lower concentration of kanamycin would allow for more non-transgenic plants to be regenerated. Since no plants carrying *chiB* could be regenerated it is evident that the concentration of kanamycin was not a factor affecting the transformation frequency in tobacco.

3.2.6 Effect of *chiB* on tumor formation

One implication of the small percentages of plants regenerated from the tobacco transformation undertaken with *chiB* is that the presence of the gene prevents or inhibits the transformation process itself rather than regeneration of plants. As far as is known, this has not been observed with any of the other bacterial or plant chitinase genes. The possibility that the *chiB* gene inhibits plant-bacterial interaction was tested by examining the effect of *chiB* on tumorgenesis by *A. tumefaciens*.

3.2.6.1 Tumor formation by *A. tumefaciens* C58

To study the possibility that the product of *chiB* prevents tumor and callus formation by *A. tumefaciens*, pAW446 was transferred to the wild-type *A. tumefaciens* strain C58. Prior to this experiment being undertaken *A. tumefaciens* C58 was confirmed

as having the ability to form galls at a wound site by inoculating a cut in the stem of a tobacco plant with 50 μL of a culture with an OD of 1 (A_{595}). Within two weeks of inoculation swelling around the wound was apparent and within 4 weeks the formation of a gall on the stem of the plant could be observed and, as illustrated in Fig.3.4, three months after inoculation a gall with a diameter of approximately 0.7 cm was present on the tobacco stem.

Once it was confirmed that the *A. tumefaciens* strain C58 has the ability to form tumors, it was transformed with the constructs pAW446 (vector with *chiB*) and pHS723 (vector alone) and the presence of the plasmids in C58 were confirmed by restriction enzyme digests and PCR. Cultures of C58[pAW446] and C58[pHS723] were grown to an OD of 1 (A_{595}) and 50 μL of each culture was used to inoculate, in duplicate, cuts on the stems of tobacco, tomato, *Zinnia elegans* and *Kalanachoe daigremontiana* plants. The plants inoculated were examined weekly for swelling around the wound sites and gall formation. As illustrated in Figures 3.5a, b, c and d, approximately four weeks after the wounds were inoculated with C58[pHS723] swelling and some gall formation were present on the stems of all of the plants. Twelve weeks following inoculation all of these plants showed definite gall formation. However, the wounds inoculated with C58[pAW446] showed no signs of swelling or gall formation (Fig. 3.5 a, b, c and d) and even 12 weeks after inoculation galls had not formed at the inoculated wound sites on any of the plants. These results supported the belief that the presence of *chiB* is inhibits tumor formation.

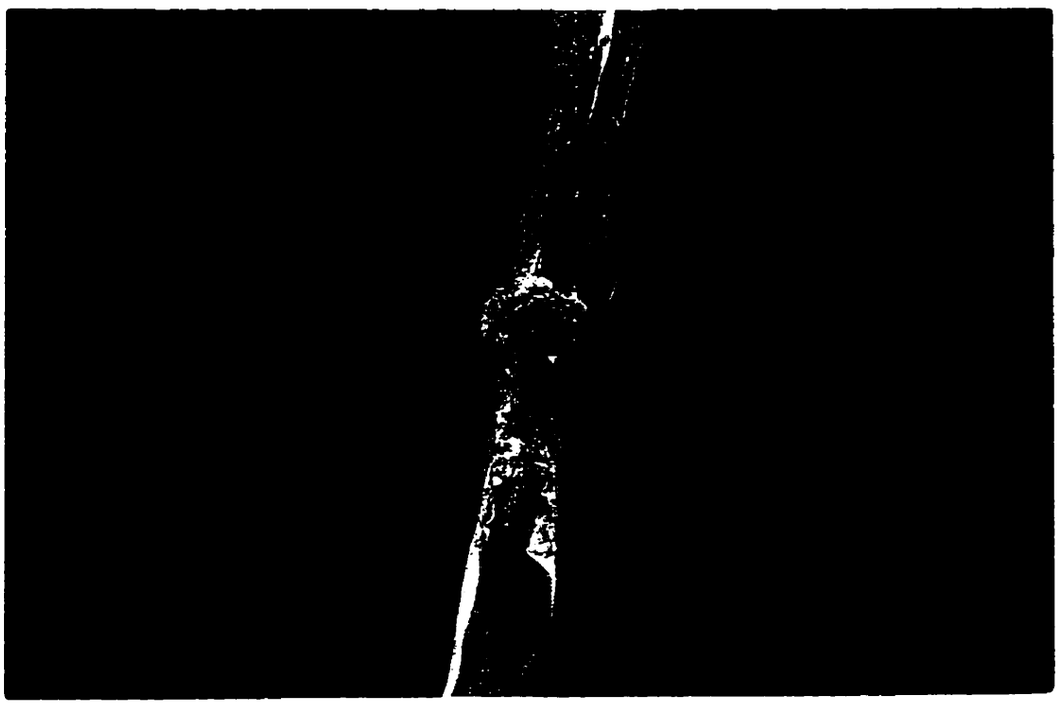

Figure 3.4. Gall formation on the stem of a tobacco plant three months after being inoculated with *A. tumefaciens* C58.

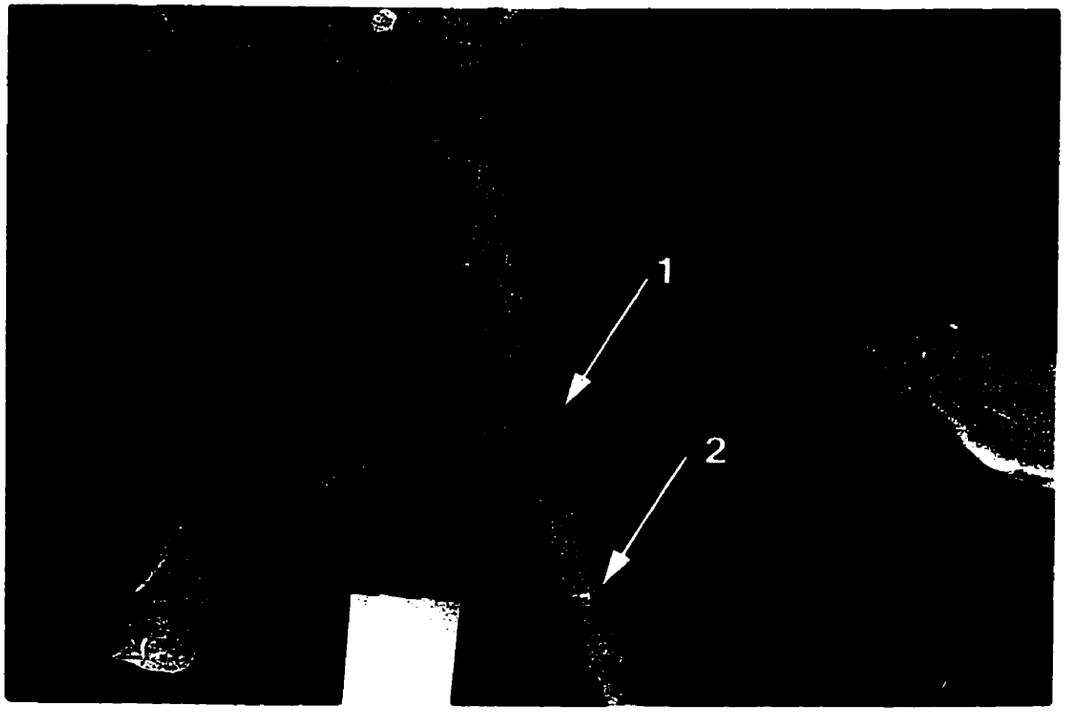

Figure 3.5a. A tobacco plant two weeks after being inoculated with the *A. tumefaciens* strains C58[pHS723] (1) and C58[pAW446] (2). Early signs of the formation of disorganized tissue is only apparent around the wound site inoculated with the strain carrying the construct which lacks the *chiB* gene.

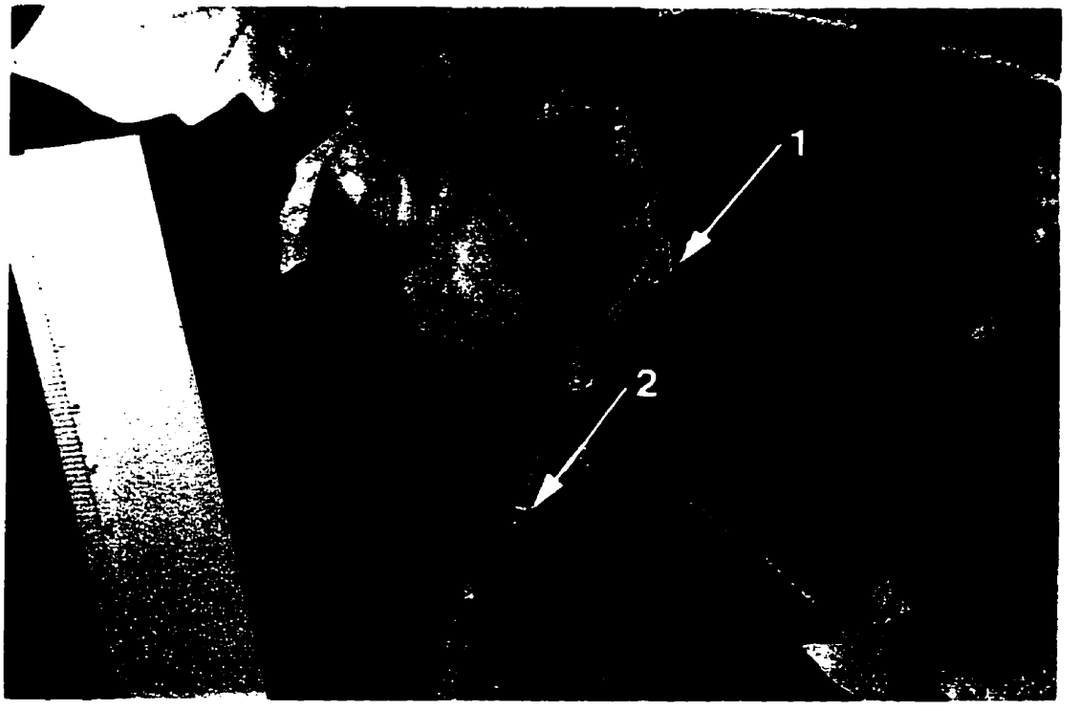

Figure 3.5b. A tomato plant stem six weeks following inoculation with the strains *A. tumefaciens* C58[pHS723] (1) and *A. tumefaciens* C58[pAW446] (2).

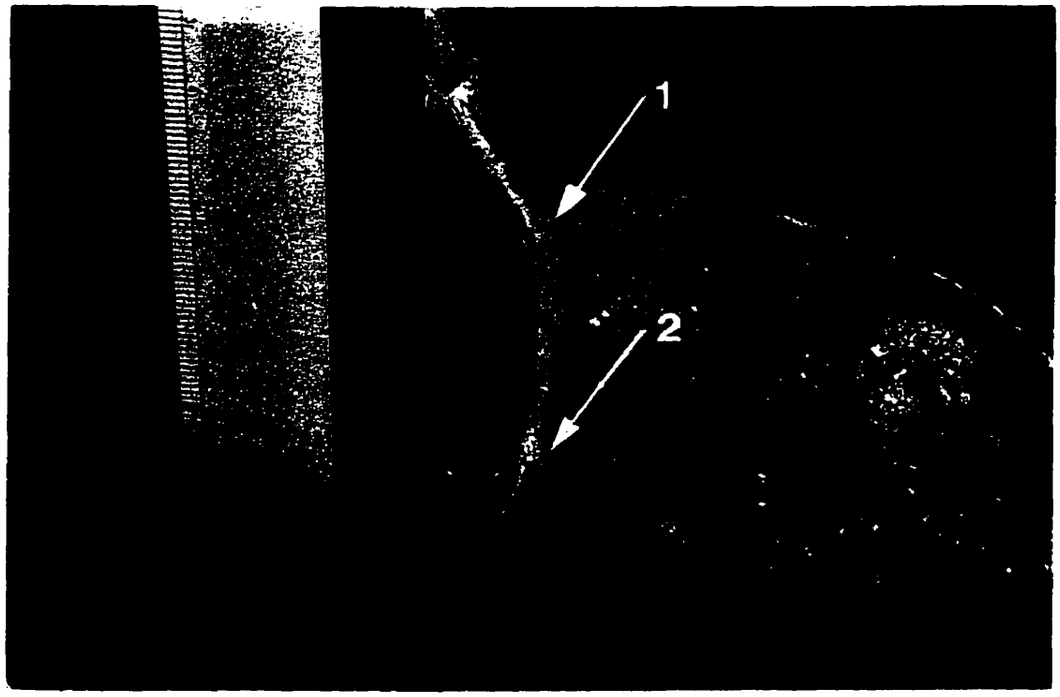

Figure 3.5c. A *Zinnia elegans* plant stem six weeks following inoculation with the strains *A. tumefaciens* C58[pHS723] (1) and *A. tumefaciens* C58[pAW446] (2).

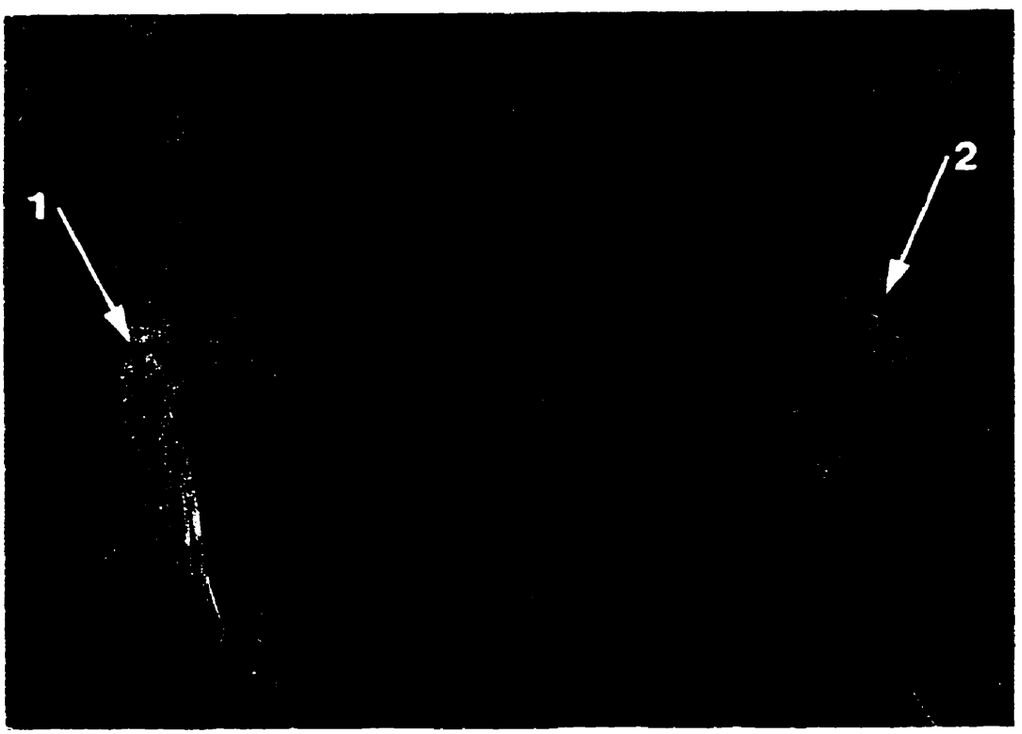

Figure 3.5d. *Kalanchoe daigremontiana* plants six weeks following inoculation with the strains *A. tumefaciens* C58[pHS723] (1) and *A. tumefaciens* C58[pAW446] (2).

3.2.6.2 Inhibition of *chiB* expression allows calli to form

Since *A. tumefaciens* strains carrying *chiB* appear to lose their ability to form tumors, a study was undertaken to determine if a functional *chiB* gene was essential for the effect. The *chiB* sequence contains two *Bam* HI sites that are approximately 800-bp apart (Fig. 3.6). To prevent the expression of *chiB* the plasmid pAW444 was digested with *Bam* HI, the 800-bp fragment dropped out and the plasmid religated. The plasmid pAW446 could not be used directly as the vector contains a *Bam* HI site. The altered *chiB* sequence was cloned into pBI524-14 to give rise to the plasmid pAW447. To ensure that the production of chitinase from *chiB* had been inhibited a chitinase assay was performed using proteins extracted from DH5α[pAW447]. As illustrated in Table 3.6, the production of chitinase was inhibited by deleting a portion of the gene. The expression cassette was then dropped out as a *Xba* I fragment and cloned into pHS723. The resulting plasmid, designated pAW448, was transferred to *A. tumefaciens* GV3101 and its integrity confirmed by restriction enzyme digests and sequencing. GV3101[pAW448] was then used to infect 20 sterile tobacco discs. The results from this experiment clearly illustrated

that the loss of chitinase activity affected the transformation process as the infected discs did not turn yellow and die as in the previous transformations. Instead, all of the discs remained green and showed signs of swelling two or three days after infection and remained green. Within four weeks of the discs being transferred to selection media calli were present on the edges of the majority of the discs.

The possibility that the *chiB* gene sequence was responsible for the data reported was extended by another line of experimentation. Expression of ChiB was also inhibited by introducing a frame shift mutation approximately two-thirds downstream from the

ATG TCC GAA CGT AAA GCC GTT ATT GTT TAT TAT TTT ATT CCG ACC AAC CAA ATT AAT AAC 60
TAT ACC GAG TCT GAT ACT TCA ATC GTG CCC TTC CCG GTG TCC AAT ATC ACA CCG GCC AAA 120
GCC AAA CAG CTG ACC CAC ATT AAT TTC TCG TTC CTC GAT ATT AAC GCC AAT CTG GAA TGC 180
GCC TG**G GATCC**C GCC ACT AAT GAC GCC AAG GCG CGT GAC GTG GTC GGC CGG TTA ACC GCG 240
CTG AAG GCA CAC AAC CCC AGC CTG CGG ATC ATG TTC TCC ATC GGC GGC TGG TAT TAC TCC 300
AAC GAT CTG GGG GTT TCC CAT GCC AAC TAT GTC AAC GCG GTG AAA ACC CCG GCG GCG CGG 360
ACC AAA TTC GCG CAA TCC TGC GTA CGC ATT ATG AAA GAC TAC GGT TTT GAC GGC GTG GAT 420
ATT GAC TGG GAA TAT CCG CAG AGC AGT GAA GTG GAC GGT TTT GTC GCC GCG CTG CAG GAG 180
ATC CGC ACC CTG CTG AAC CAG CAA ACC CTG GCT GAC GGT CGC CAG GCG CTG CCT TAC CAA 540
CTG ACA ATT GCC GGT GCC GGT GGC GCT TTC TTC CTG TCG CGC TAC TAC AGC AAG CTG CCG 600
CAG ATC GTC GCC TCA CTC GAT TAC ATC AAC CTG ATG ACC TAC GAT CTG GCC GGC CCT TGG 660
GAG AAA ATC ACC AAC CAC CAG GCG GGA CTG TTC GGC GAC AGC GCC GGG CCA ACC TTC TAT 720
AAC GCG CTG CGC GAA GCC AAC CTG GGC TGG AGC TGG GAA GAA CTG ACC CGC GCC TTC CCA 760
AGC CCC TTC AGC CTG ACG GTT GAT GCC GGC GTG CAG CAG CAC CTG ATG CTG GAA GGC GTG 820
CCG AGC AAC AAG ATC GTC ATG GGC GTG CCG TTT TAT GGT CGT GCG TTC AAG GGC GTC AGC 880
AGC AGT AAC GGC GGC CAG TAC AGC AGT CAC AGC ACG CCG GGG GAA GAT CCG TTC CCG GGC 940
ACC GAC TAC TGG CTG GTG GGT TGC GAC GAA TGT GTC CGC GAC AA**GGATCC**G CGC ATC GCC 1000
TCC TAC CGC CAA TTG GAG CAA ATG CTG CTC GGC AAC TAC GGC TAT CAG CGC CTG TGG AAC 1060
GAC AAG ACC AAA ACT CCG TAC CTG TAT CAC GCG GCC AAC GGC CTG TTC GTT ACC TAT GAC 1120
GAT GTC GA**A AGCTT**C AAG TAC AAG GCG AAG TAC ATC AAG CAG CAG CAA CTG GGC GGC GTG 1180
ATG TTC TGG CAT CTG GGC CAG GAT AAC CGT AAC GGT GAC CTG CTG GCG TCG CTG GAC CGC 1240
TAT TTC AAC GCG GCG GAC TAC GAT GAC AGT CAA CTG GAT ATG GGC ACC GGC CTG CGT TAT 1300
ACC GGC GTG GGT CCG GGT AAT TTG CCA ATC ATG AGT GCC CCG GCC TAC GTC GCC GGT ACC 1360
ACC TAT AAC CAG GGA GCG CTG GTG TCT TAT CTT GGC TAC GTC TGG CAG ACC AAG TGG GGC 1420
TTC ATC ACT TCC GTA CCC GGC TCG GAC AGC GCC TGG CTG AAA GTG GGC CGC GTA GCA TAA 1480
CGG TAG

Figure 3.6. Nucleotide sequence of the *chiB* gene of *S. liquefaciens*. The *Bam* HI and *Hind* III sites used to create the deletion and frame shift mutants are underlined and shown in bold type.

start codon. The frameshift mutation should disrupt the production of a functional chitinase in spite of the fact that the entire sequence of the gene is present. This was done because by not removing a portion of the gene sequence it should be possible to distinguish if the expressed protein inhibits transformation or if a specific sequence within the gene causes the transformation process to be halted. Since the *chiB* gene possesses a single *Hind* III at 1148 nt 3' to the initiation codon, pAW444 was cut with *Hind* III, filled-in with Klenow polymerase fragment of polymerase I using dNTPs and religated resulting in the construct pAW460. This manipulation adds four nucleotides at this site causing a translational frameshift while keeping the nucleotide sequence readily intact. Radiochemical chitinase assay was then undertaken on the crude protein extracts isolated from DH5α[pAW460]. The results obtained from this part of the study, shown in Table 3.6, confirmed that the mutated gene does not produce chitinase.

The expression cassette, with the introduced frameshift mutation, was dropped out as a *Xba* I fragment and cloned into pHS723. The resulting construct, pAW461, was transferred to *A. tumefaciens* GV3101 and its presence confirmed by restriction endonuclease digests. Tobacco discs were infected with this construct and as a control pAW446. The loss of chitinase activity due to the frameshift mutation had a definite effect on the formation of calli as the infected discs remained green and were found to swell after infection. Three weeks after the discs had been transferred to selection media calli started to form on the majority of the discs. However, those discs infected with the *A. tumefaciens* strain carrying pAW446 did not swell normally and after five weeks all

Table 3.6 Chitinase activity in bacterial protein extracts

Protein Extract	Status of *chiB* sequence	^{3}H chitin cpm solubilized per protein reaction) [1]. 10 μg protein
DH5α [pAW444]	Functional *chiB* sequence	2446
DH5α [pAW447]	Deletion mutation	593
DH5α [pAW460]	Frameshift mutation	846
Control (no protein)	Not applicable	735

[1] The chitinase assay of Molano *et al.* (1977) was used to determine the ^{3}H counts released into the soluble fraction from the insoluble tritiated chitin used as the substrate. Each of the values is the average of the results obtained in two separate experiments.

had turned yellow and failed to form calli as shown in Figure 3.7.

3.2.6.3 Inhibition of *chiB* expression allows for the formation of galls

To determine whether or not the inhibition of *chiB* expression allowed for the formation of galls around the wound sites tobacco and tomato plants were inoculated with the *A. tumefaciens* C58 strains carrying the constructs pAW448 and pAW461. In this experiment, 50 μL of each culture (OD = 1 at A_{595}) was used to inoculate a cut in the stem of the plants. Approximately six weeks after inoculation swellings were observed around the wound sites of the plants and by nine weeks tumors started to form, which indicates that when the expression of *chiB* is inhibited by the introduction of a frameshift normal tumor formation occurs as shown in Figures 3.8 a and b. Plants inoculated with C58[AW446] did not develop tumors as expected.

This experiment was then taken one step further. Competent cells of the *A. tumefaciens* strains C58[pAW446], which lack the ability to form galls, and C58[pHS723] were prepared and transformed with a broad host range plasmid known as pHS63 which contains the same replicon as pHS723 and pAW446. As the plasmid pHS63 is not compatible with either of the plasmids pAW446 or pHS723, selection for its presence would prelude coexistence of either of these plasmids. Since pHS63 contains the selectable marker spectinomycin it was possible to select for the presence of pHS63 and screen for the loss of the other plasmid that carried kanamycin resistance. After streaking colonies onto 2YT plates containing spectinomycin for several generations the loss of the plasmids pAW446 and pHS723 and the presence of pHS63 were confirmed by extracting plasmid DNA and performing restriction enzyme digests. The two strains

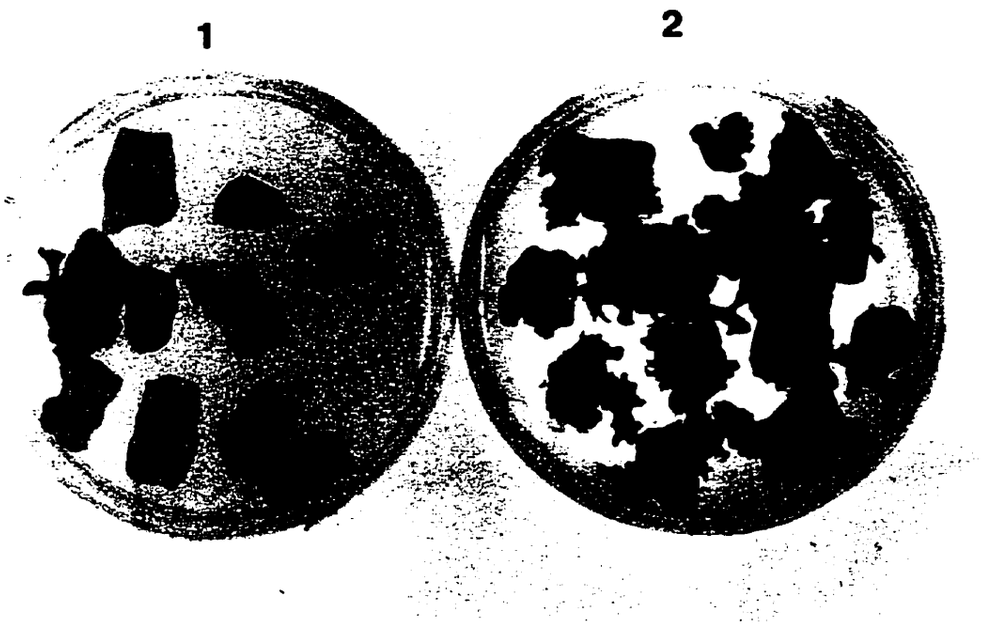

Figure 3.7. A comparison of tobacco discs infected with *A. tumefaciens* GV3101[pAW446] (1) and *A. tumefaciens* GV3101[pAW461] (2) incubated on co-cultivation media and selection/shooting media for three days and four weeks, respectively.

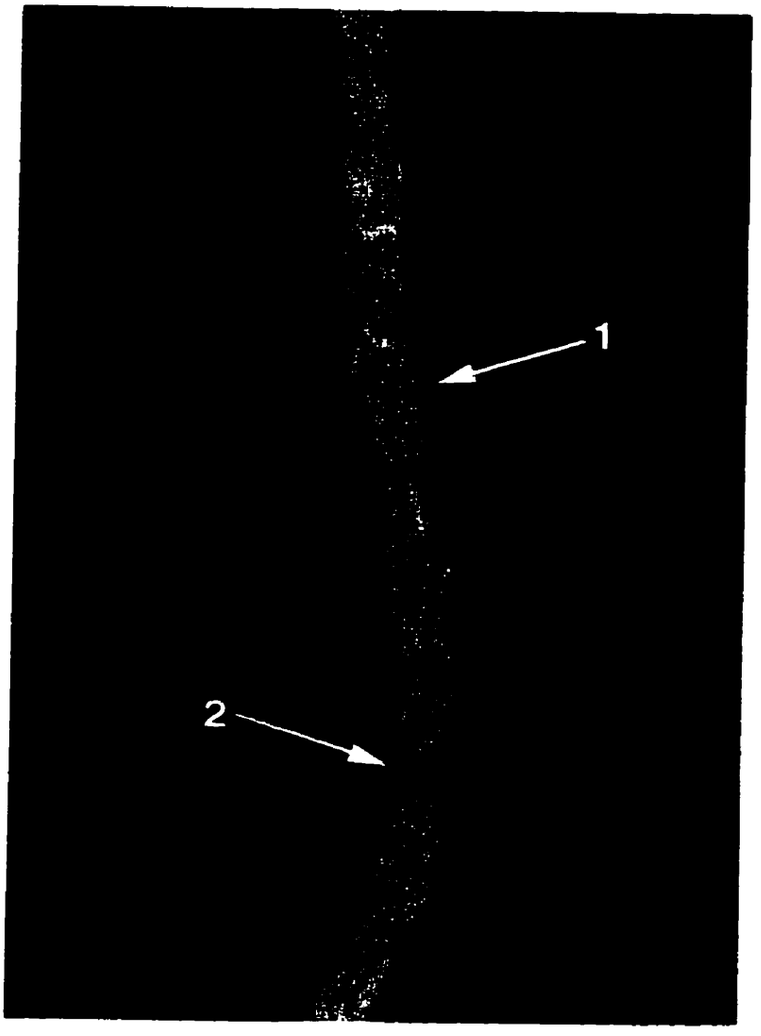

Figure 3.8a. The formation of galls on the stem of a tobacco plant three months after inoculation with *A. tumefaciens* C58[pAW448] (1) and *A. tumefaciens* C58[pAW461] (2).

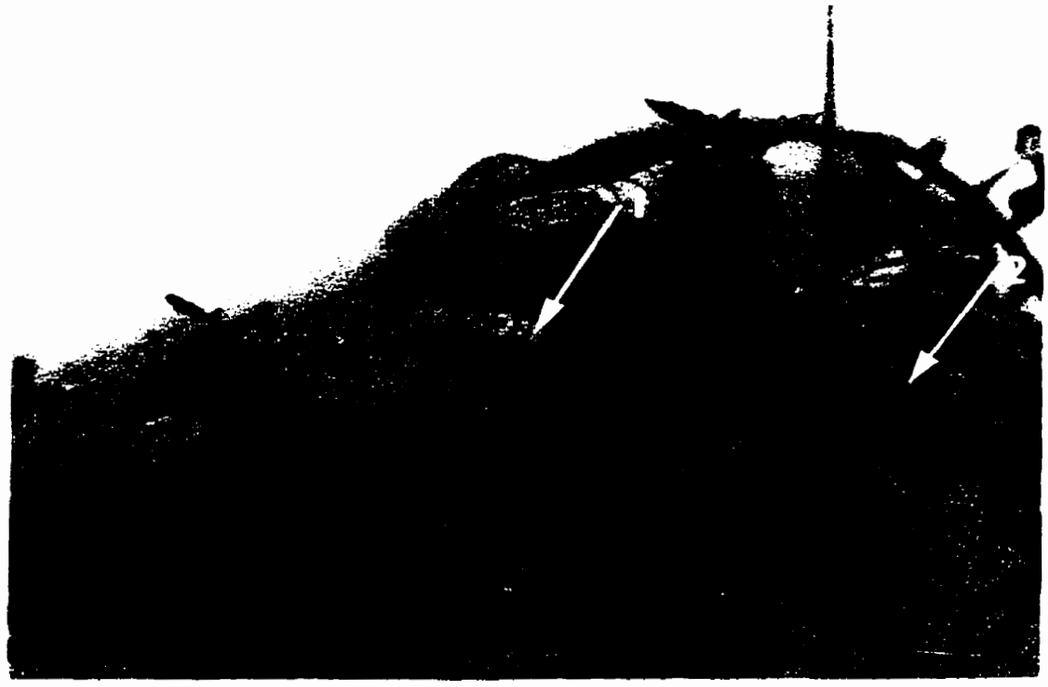

Figure 3.8b. The beginning of gall formation on the stem of a tomato plant five weeks after inoculation with *A. tumefaciens* C58[pAW448] (1) and *A. tumefaciens* C58[pAW461] (2).

carrying pHS63 rather than pAW446 and pHS723 were used to inoculate *Kalanchoe blossfeldiana* plants. Approximately six weeks after inoculation swelling and the start of tumor formation could be seen on the stems of each of the plants as shown in Figure 3.9. These results also showed that tumor formation occurs when *chiB* is absent from the *Agrobacterium* strain used to inoculate a wound site.

3.2.7 Delaying the application of *chiB* to an inoculated wound leads to the inhibition of tumor formation

Another possibility is that the chitinase encoded by *chiB* may inhibit some type of tumor inducing signal produced by the bacteria and recognized by the plant cell. To examine such a possibility *K. blossfeldiana* plants were wounded and then inoculated with 50 µl of the cultures *A. tumefaciens* 281 and *A. tumefaciens* 348 (OD of 1 at A_{595}). The inoculum was allowed to enter the plant cells for 24 hours after which each site was reinoculated with 50 µL of either the strain *A. tumefaciens* C58[pAW446] or *A. tumefaciens* C58[pHS723] (OD of 1 at A_{595}). The plants were then examined daily for the formation of tumors. Approximately four weeks after inoculation galls started to form only on the plants which had been inoculated with strains 348 and 281 and then C58[pHS723]. No visible changes were observed on the wound sites that had been reinoculated with C58[pAW446] (Fig. 3.10 a, b and c). The plants were observed for another two months, but no gall formation was observed on the C58[pAW446] inoculated plants. Thus, it appears that whenever the chitinase gene is present, even 24 hours after inoculation of wildtype *Agrobacterium* strains, tumor formation is halted.

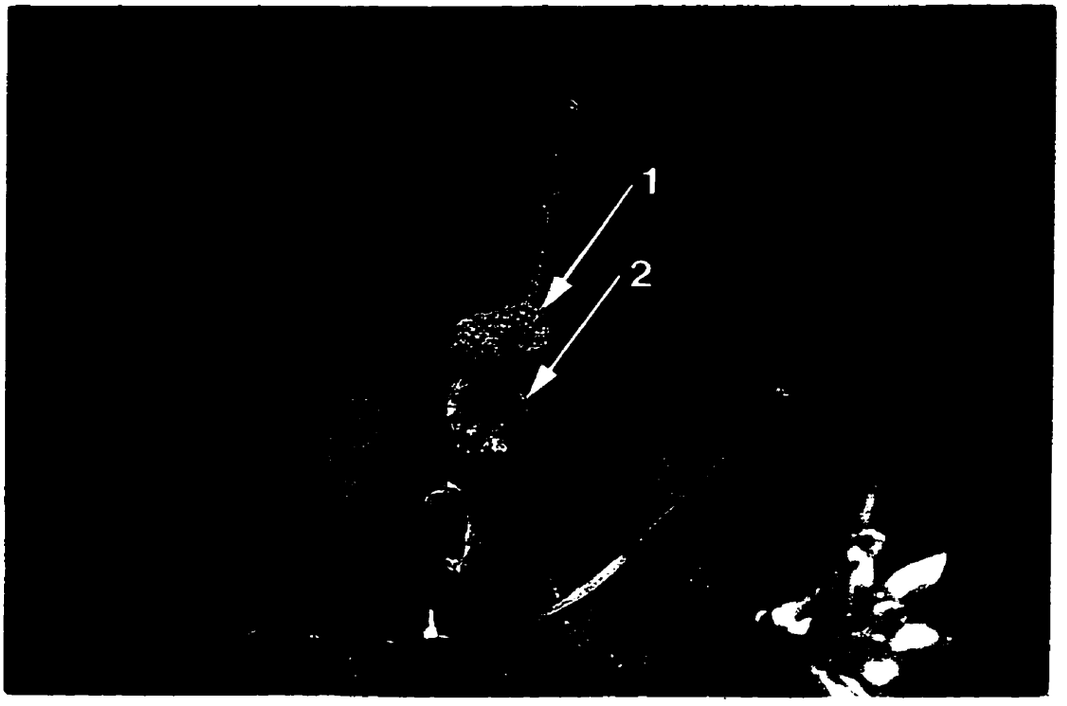

Figure 3.9. The formation of galls on the stem of a *Kalanchoe blossfeldiana* plant eight weeks after inoculation with *A. tumefaciens* GV3101[pAW446/pHS63] (1) and *A. tumefaciens* GV3101[pAW723/pHS63] (2).

Figure 3.10a. The formation of galls on the stem of a *Kalanchoe blossfeldiana* plant four weeks after infection with *A. tumefaciens* 348 (1) and *A. tumefaciens* 281 (2) followed by *A. tumefaciens* GV3101[pHS723] 18 hours later.

Figure 3.10b. Inhibition of gall formation on the stem of a *Kalanchoe blossfeldiana* plant four weeks after infection with *A. tumefaciens* 348 followed by *A. tumefaciens* GV3101[pAW446] 18 hours later.

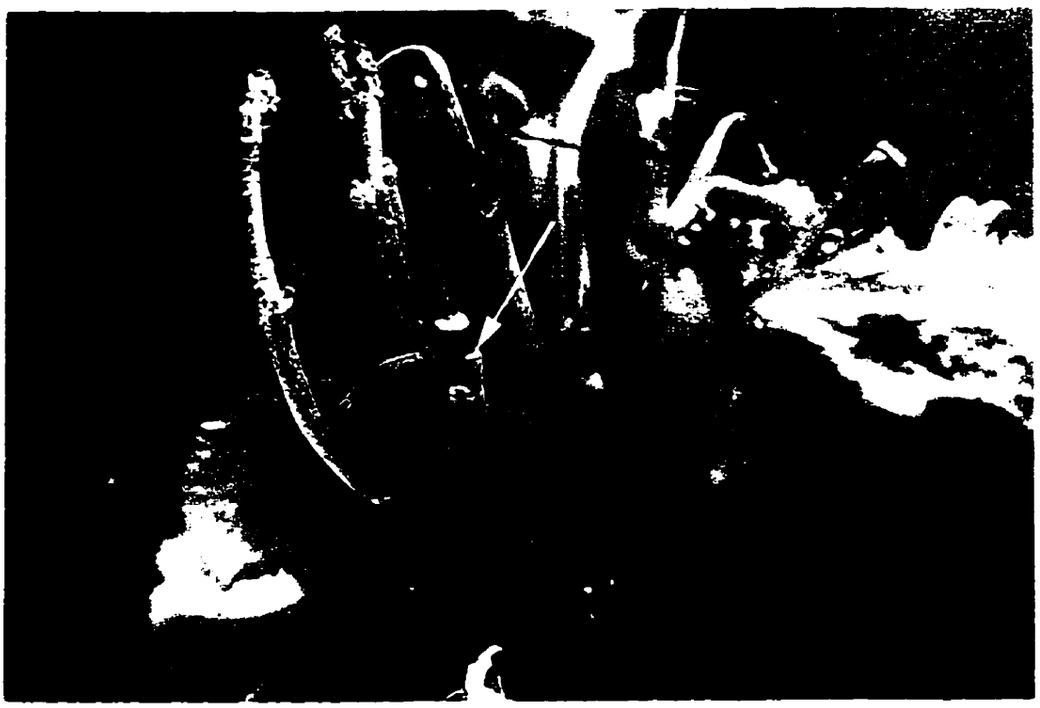

Figure 3.10c. Inhibition of gall formation on the stem of a *Kalanchoe blossfeldiana* plant four weeks after infection with *A. tumefaciens* 281 followed by *A. tumefaciens* GV3101[pAW446] 18 hours later.

3.2.8 Determining if *Agrobacterium tumefaciens* C58 contains a gene that shares homology to the *nodC* gene of *Rhizobium meliloti*

To determine if there is a gene present in the genome of *A. tumefaciens* C58 that shares significant homology to the *nodC* gene of *R. meliloti* Southern blot analysis was performed on DNA extracted from *A. tumefaciens* C58 and digested with *Eco* RI. The probe used to undertake the Southern blot analysis was obtained by amplifying a 900-bp fragment of the 5' end of the *nodC* gene using PCR. The membrane was probed with the radiolabelled probe at different hybridization temperatures and washes. However, in each case no bands appeared on the autoradiogram thus suggesting that the *A. tumefaciens* strain C58 does not contain a gene that shares significant homology to this portion of the *nodC* gene.

3.3 Discussion

It has been known for a number of years that microbes exploit plants as favorable niches for colonization. Approximately 80 species of bacteria are known to interact with plants and some of these interactions are beneficial to plants while others are detrimental. Depending on the microbes penetrating the plant tissue the consequences of colonization can vary from asymptomatic, to symbiosis or to disease. To deal with such challenges plants have evolved elaborate defense systems that exclude many microorganisms from colonizing within their tissues. Generally, there are three defense responses that occur in plants and they include accumulation of structural host-cell wall proteins and secondary metabolites, accumulation of host antibiotics, and production of hydrolytic enzymes and other antimicrobial proteins (Graham and Graham, 1991). However, certain microbes have also evolved defenses and have the ability, therefore, to penetrate and colonize plant tissue. Bacteria belonging to the *Rhizobiaceae* family provide a good example of a complex plant-bacteria interaction that leads to minimal plant cell death within the plant (Baron and Zambryski, 1995).

The original purpose of this study, as has already been stated, was to determine if it was possible to produce tobacco and canola plants with an increased resistance to certain plant pathogens by the introduction of a chitinase gene isolated from *S. liquefaciens* alone, and in combination with other antimicrobial genes, into their genomes. However, as this study progressed it became apparent that the presence of the *chiB* somehow inhibited the *A. tumefaciens*-mediated genetic transformation, as evidenced by a lack of recoverable transgenics despite many attempts. After a number of

unsuccessful tobacco and canola transformations it was decided that this study should focus on determining if *chiB* affected *Agrobacterium*-plant interactions.

In order for the chitinase-encoding sequence from *S. liquefaciens* to be transferred and expressed in tobacco and canola plants it was placed under the control of an enhanced 35S CaMV promoter within a disarmed T-DNA. To clone *chiB* into an expression cassette so that it would be in the correct reading frame a *Nco* I site had to be added just prior to the ATG start codon. The addition of this restriction site made it necessary to add an extra amino acid immediately following the methionine. Radiochemical chitinase assays were undertaken to ensure that the additional codon present at the 5' end of the gene did not alter the expression of *chiB*. The results from this experiment indicated that the altered gene produced a protein with strong chitinase activity. The expression cassette was cloned into the plant vector pRD400 and the resulting construct, pAW445, was then used to carry out several tobacco and canola transformations. In the first tobacco transformation undertaken, using the strain *A. tumefaciens* GV3101[pAW445], 100 leaf discs were infected but only 15 plants were regenerated. Since tobacco is a plant which is transformed easily with *A. tumefaciens* transformation frequencies of 80% or higher are usually observed. Therefore, when a transformation frequency of only 15% was observed the result was very surprising and perplexing. Furthermore, PCR and Southern blot analysis undertaken on the 15 plants regenerated confirmed that none of them had *chiB* integrated into their genomes. Tobacco discs were infected with the same strain of *A. tumefaciens* for a second time and no shoots developed from either transformation. A summary of the transformations

undertaken with the *chiB* sequence is presented in Table 3.7. It was confirmed that the strain being used for each transformation was viable as a thin film of growth could be observed around each of the infected discs following incubation on co-cultivation media. However, it was noted that the infected discs did not swell as much as discs infected with the strains carrying different gene constructs. Approximately two weeks after the discs had been placed on selection media it was found that all of the discs began to yellow and failed to form calli. From the findings observed from these two transformations it appears as if the T-DNA was not transferred to the plant cells because when uninfected tobacco discs were placed on selection media they swelled very little, turned yellow, failed to produce calli and eventually died.

While continuing the work with the GV3101[pAW445] transformations a new construct, designated pAW446, was made using the plant vector pHS723. This vector is very similar to pRD400 except that it has the GUS gene which allows for quick screening of potential transgenic plants. Once the expression cassette had been cloned into pHS723 and the presence of *chiB* confirmed, several *Agrobacterium*-mediated tobacco and canola transformations were performed. In the case of the canola transformation no plants were regenerated. In the tobacco transformation an additional 100 infected discs were incubated for an additional four days on co-cultivation media to see if discs which had been in contact with the bacterial cells for a longer duration were successfully transformed. In this experiment it was found that shoots did not form on any of the discs which had been incubated for only three days on co-cultivation media but some calli,

Table 3.7 Summary of transformations undertaken and the results obtained with the *chiB* sequence

Transformation	Number of discs or cotyledons infected	Number of plants regenerated	Number of *chiB* plants
Tobacco-pAW445	100	15	0
Tobacco-pAW445	200	0	0
B. napus-pAW445	2000	0	0
B. napus-pAW445	3000	0	0
B. napus-pAW445	5000	0	0
Tobacco-pAW446	200	12	0
Tobacco-pAW446	200	32	0
Tobacco-pAW446	200	0	0
Tobacco-pAW446	200	0	0
Tobacco-pAW446	200	8	0

although slow in developing, did form on some of the discs which had been on co-cultivation media for a longer period of time. The regeneration frequency from these discs was also very small as only 12 shoots developed. GUS activity was monitored in the 12 plantlets as they began to develop roots. Seven of these plantlets were determined to be positive for GUS activity and this activity was further tested at different stages of growth such as the four leaf, eight leaf and flowering stages. While monitoring GUS activity in these plants at various stages of development it was found that all of them displayed strong GUS activity while rooting and upon transfer to potting soil, but as each plant matured the GUS activity steadily decreased until little or no activity was observed in the leaf tissue assayed. PCR and Southern blot analysis carried out on DNA extracted from the seven GUS positive plants indicated that all of them were negative for the integration of *chiB*. When a second tobacco transformation with GV3101[pAW446] was undertaken a transformation frequency of approximately 30% was observed. In this transformation 32 plants were regenerated of which 22 were found to be positive for GUS activity. However, when the leaves assayed for GUS activity were examined under a stereomicroscope it was found that most of the coloration appeared to be localized on the leaf surfaces and hairs, which suggests that the GUS activity occurring in these plantlets was probably due to the presence of residual *A. tumefaciens* cells. These plants, as with the plants regenerated from the first transformation, were monitored for GUS activity as they matured and in each plant the activity was found to steadily decrease until no GUS activity could be detected. Results from PCR and Southern blot analysis performed on DNA isolated from the GUS positive plants revealed once again that *chiB* had not integrated into their genomes.

Although the GV3101[pAW446]-mediated transformations carried out in this study produced a high number of false positive transformed tobacco plants it should be noted that such an observation was not completely unexpected. Since *A. tumefaciens* are motile bacteria it is assumed that if given the chance they will move throughout a plant. For example, in grapevine where the bacteria colonize the vascular tissue and spread systemically, *A. tumefaciens* moves through the cutin-free cortex tissue, rhizodermis and vascular system by swimming through intercellular spaces (Lehoczky, 1968; Stellmach, 1990). Such latent infections with *A. tumefaciens* do not cause disease-symptoms but rather demonstrate that *A. tumefaciens* cells are capable of living and multiplying within various tissues of their host plants. In all of the transformations performed in this study carbenicillin was incorporated, at a concentration of 500 mg/l, into the selection, shooting and rooting media to eliminate any residual *A. tumefaciens* that may have been present. Unfortunately, in many cases the complete elimination of *A. tumefaciens* from transgenic tissue was found to be difficult. This was probably due to the antibiotics being bacteriostatic instead of bactericidal (Mogilner *et al.*, 1993; Landsmann *et al.*, 1995). If residual bacteria do remain within an untransformed plant gene expression by the *A. tumefaciens* cells which remain attached to inoculated tissues is always a possibility. However, this study showed that residual bacteria decrease as the plant grows since GUS activity steadily decreased as the plants continued to grow and mature. As well, chitinase assays undertaken with proteins extracted from the various "GUS positive" plants indicated that there was no significant increase in chitinase activity in these plants when compared to control plants.

A recent study undertaken by Matzk and co-workers (1996) on the localization of persisting agrobacteria in transgenic tobacco plants has confirmed that *A. tumefaciens* cells containing a binary vector can persist for at least 18 months post-transformation in both transgenic *in vitro* and *ex vitro* plants. Furthermore, this group was able to prove unequivocally that *A. tumefaciens* cells survive the standard antibiotic treatments of cefotaxime and carbenicillin even when applied to the tissue culture medium for 12 months after initial infection. These observations are in agreement with those of Hoeven *et al.*, (1991) who found persistence of *A. tumefaciens* in transformed plants for a few months post-transformation. It is results such as these that would explain why so many of the tobacco plants regenerated in this study were initially positive for GUS activity, but negative for the integration of *chiB* into their genomes. It is interesting to note that although the GUS assays were positive, the presence of *chiB* was never detected by either PCR or Southern blot analysis. The most logical reason for this observation is that older leaf tissue, which was found not to display strong GUS activity, was used to extract plant DNA. As well, no type of selective enrichment for the presence of *A. tumefaciens* from macerated plant material was employed while extracting the DNA. In the study undertaken by Matzk *et al.* (1996), it was found that while *A. tumefaciens* tends to be uniformly distributed throughout *in vitro* plants it is only present at the basal positions of *ex vitro* plants. In this study, similar findings were also observed with the *chiB* transformations since GUS activity was highest in the newly generated shoots and very young plants, but as they matured the activity was found to drop significantly until no activity was observed in either the flowers or seeds.

Tobacco leaf discs were infected with the strain GV3101[pAW446] for a third and fourth time and in both cases the discs yellowed and failed to form calli or shoots. The presence of the vector and *chiB* in the *A. tumefaciens* strain used for all of the transformations was confirmed several times by PCR and restriction enzyme digests. To ensure that the vector pHS723 was not the source of the problem several transformations were done in parallel with GV3101[pAW446]. In every transformation the discs infected with the GV3101[pHS723] became swollen and formed calli and shoots. The different components used in the tissue culture media were also examined to determine if any particular one might be the source of the problem. Different lot numbers of MS media, antibiotics and hormones were individually tested and eventually all of the components examined were ruled out as possible inhibitors due to the fact that control transformations, done at the same time, continued to be successful but not the *chiB* transformations. Since none of the components used in preparing the media required for the tobacco transformations acted as inhibitors, the concentration of kanamycin employed was examined to see if it might be too high. When the concentration of kanamycin was lowered to 50 μg/mL it was found that approximately 80% of the discs cultivated with GV3101[pAW446] formed calli and developed shoots. Further tests, however, revealed that none of the regenerated plants contained *chiB*. The lower concentration of kanamycin employed in the tissue culture media of this experiment would allow for more non-transgenic plants to be regenerated. Therefore, the observed results were to be expected and it can be concluded that the concentration of kanamycin was not a major factor adversely affecting the transformation process. Finally, it was concluded that the presence of the *chiB* gene product must have an adverse effect on the *Agrobacterium*-mediated

transformation process and prevent the transfer and integration of the gene into the plant cells.

Observations presented in this dissertation are unique. Although there are many other research groups undertaking *Agrobacterium*-mediated transformations using either a bacterial or plant chitinase gene no one has reported such a phenomenon. Chitinase genes from a number of different bacteria and plant species have been isolated and successfully introduced into different species of plants. For example, one of the first chitinase genes to be successfully transferred to tobacco was the *chiA* gene of *Serratia marcescens* (Dunsmuir and Suslow, 1989). This research group was able to establish homozygous tobacco lines with a single *chiA* locus and examination of plants that produced high levels of ChiA were found to be more resistant to infection by the tobacco brown-spot pathogen *Alternaria longipes*. Transgenic tobacco and canola plants, which constitutively express the bean chitinase gene, have also been regenerated and found to have greater resistance to the development of disease when challenged with the phytopathogen *Rhizoctonia solani* (Broglie *et al.*, 1991). More recently, a chitinase gene from *Lycospersicon chilense* has successfully been transferred to tomato plants (Agharbaoui *et al.*, 1996). A chitinase gene isolated from petunia has also been transferred to carrot and cucumber using the *Agrobacterium*-mediated transformation process. Since other bacterial and plant chitinases have been successfully transferred to plants the basic question arising from the results of this study is what causes the *chiB* gene product of *S. liquefaciens* to inhibit the *Agrobacterium*-mediated transformation process.

To determine whether or not the *Agrobacterium* strains carrying the *chiB* construct lack the ability to form tumors around inoculated wound sites different plant species were inoculated with *A. tumefaciens* C58[pAW446] and the control strain *A. tumefaciens* C58[pHS723]. Examination of plants inoculated with the two strains revealed that the control strain was oncogenic, but the strain carrying *chiB* was not. The *A. tumefaciens* strains C58[pAW446] and C58[pHS723] were also transformed with and selected over several generations for a broad host range plasmid, known as pHS63, to determine if the oncogenic activity of GV3101[pAW446] returned once the chitinase construct was removed and replaced with a similar plasmid. In this experiment galls were found to develop very quickly on the stems of *K. blossfeldiana* plants that had been inoculated with GV3101[pAW446/pHS63] and GV3101[pHS723/pHS63]. These results confirmed that the specific *A. tumefaciens* strain that has continuously harbored the plasmid containing *chiB* does have the ability to form galls when the plasmid is removed and replaced with a similar plasmid.

To investigate further all of the findings observed in the various experiments undertaken with the *chiB* construct another set of experiments was carried out using constructs in which the expression of *chiB* had been inactivated by the introduction of deletion or frameshift mutations. The deletion mutant was created by removing approximately 800-bp of the gene and chitinase assays confirmed that chitinase was not produced from this gene. When *A. tumefaciens* carrying this construct was used to inoculate wounds on the stems of tobacco plants tumors were observed to develop. Furthermore, tobacco transformations undertaken using this construct were very successful as the discs did not turn yellow when placed on selection media, but formed

calli which led to the regeneration of several transgenic tobacco plants. From this experiment it became evident that if the chitinase activity is inhibited the oncogenic ability of *A. tumefaciens* is retained. One problem with this experiment is that by removing 800-bp of the gene it is not known if a specific sequence that is present in the gene and which may be responsible for suppressing the oncogenicity is removed. To test this possibility functional *chiB* expression was also inhibited by introducing a frameshift mutation approximately 1.1-kb downstream from the start codon of *chiB*. The main advantage of using a frameshift mutation is that the entire sequence of the gene is present but there is less likelihood of the translation event being altered too early which may inhibit the formation of, for example, a required structure that may be critical for the suppression of oncogenicity. When the *A. tumefaciens* strain carrying the altered gene was used to inoculate wound sites on tobacco plants it was found that the oncogenic activity of the strain was no longer suppressed. Tobacco transformations undertaken with the inactivated gene revealed that the infected discs swelled normally and formed calli which led to the regeneration of several transgenic tobacco plants. The results obtained with this construct clearly indicated that inhibition of *chiB* had a positive effect on the oncogenicity of *A. tumefaciens* and that the suppression of oncogenicity is not likely due to a specific sequence present within the *chiB* gene.

To date no research has been published on the presence of a bacterial or plant chitinase gene that leads to suppression of oncogenicity in *A. tumefaciens*. However, it has been found that the oncogenic activity of *A. tumefaciens* can be completely abolished by the presence of a 39-kb IncW plasmid known as pSa (Loper and Kado, 1979; Vlasak and Ondrej, 1985). Since this finding Close and Kado (1991) have isolated, cloned and

sequenced the gene that encodes the protein which is responsible for suppressing oncogenicity. Although it has been demonstrated that the *osa* (oncogenic suppression activity) gene alone is sufficient for complete inhibition of oncogenicity it has been shown that it is part of an operon consisting of three other genes known as *nuc, orf-1* and *orf-3* (Chen and Kado, 1994). Currently the exact mechanism of inhibition is not known, but it has been proposed that *osa* may be involved in fertility inhibition which would prevent or reduce the formation of stable mating pairs and T-DNA transfer (Chen and Kado, 1994). A comparison of the nucleotide sequences of *chiB* and *osa* has shown that no homology exists between the two genes. Therefore, it is not likely that *chiB* inhibits the oncogenicity of *A. tumefaciens* in the same manner as the *osa* gene product.

Since *Agrobacterium* spp. are genetically related to *Rhizobium* spp. it can be speculated that some of the factors involved in the symbiotic relationship between *Rhizobium* and legumes may also play a role in the formation of galls by *A. tumefaciens*. As reviewed earlier, the formation of a gall of disorganized callus tissue is achieved by the transfer of a fragment of bacterial DNA, that directs the production of opines, to the nuclei of plants and integration of it into the genomic DNA. The opines produced in the gall then provide a source of nutrients to the colonizing bacteria. The discrete segment of bacterial DNA transferred is referred to as the T-DNA and it is present on the tumor-inducing plasmid along with other nontransferred genes which encode proteins that mediate the transfer and direct opine catabolism. As previously mentioned (Stachel and Zambryski, 1986; Zambryski, 1992), a set of *vir* genes located on the Ti plasmid code for the functions involved in plant cell recognition and provide most of the products that mediate T-DNA movement. Although root infection and the subsequent development of

legume root nodules by *Rhizobium* spp. do not involve *vir* genes specifically, this too is a multistep process that is genetically controlled by a signal exchange between the macro- and microsymbiont. As with *Agrobacterium*, the first steps in nodule formation by *Rhizobium* involve chemotaxis and attachment of the bacteria to the plant. Once the bacteria are attached to the surface of the root branching, deforming and curling occur. The substances responsible for this phenomenon in all *Rhizobium*-plant interactions studied to date are lipo-oligosaccharides, also known as Nod factors.

Research has demonstrated that a major function of the *nod* genes is to ensure signal exchange between the two symbiotic partners. The *nod*ABC genes have been shown to be sufficient to synthesize the backbone of the signal molecules which consist of 4 to 5 N-acetylglucosamine residues carrying an N-linked acyl group (Spaink *et al.*, 1993). Several studies have also shown that if any of the genes responsible for the synthesis of the lipo-oligosaccharides are mutated then nodule formation is either very poor or totally inhibited. Therefore, the lipo-oligosaccharides are a key component for the formation of nodules and structurally they are very similar to chitin which is a known substrate for ChiB of *S. liquefaciens*. From the results obtained in the research presented in this dissertation it is possible that what is being dealt with is a *A. tumefaciens*-host plant interaction that is similar to the *Rhizobium*-legume interaction that has been thoroughly studied in several different species and shown to involve the presence of chitin-oligosaccharides. Should *A. tumefaciens* require the presence of similar chitin-oligosaccharides for successful transformation and tumorigenesis to occur then it is very likely that the *chiB* gene product of *S. liquefaciens* inactivates the chitin-oligosaccharides by degrading them, thereby leading to the inhibition of callus formation and

oncogenicity. However, when *chiB* is inactivated by the introduction of a deletion or frameshift mutation the oligosaccharides are not degraded and the transformation process, and gall formation, occur without any problems.

The idea that "Nod-like factors" may be required by *A. tumefaciens* for oncogenicity and formation of calli is not an unreasonable explanation since recent research has demonstrated that the production of "Nod-like factors" is not limited strictly to Rhizobia. For example, Semino and Roberts (1995) have found that the *Xenopus* DG42 gene, which is only expressed between midblastula and neurulation stages of embryonic development, has striking sequence similarity to NodC. When DG42 was synthesized in an *in vitro* coupled transcription-translation system it catalyzed the synthesis of a wide variety of chitin oligosaccharides thus leading to the hypothesis that a Nod signaling system may operate during the early stages of vertebrate embryonic development. The possible role of chitinases and "Nod-like" factors in the rescue of mutant carrot embryos has also been studied and it has been suggested that similar factors are important in development. Research on the rescue of carrot somatic mutant embryos has revealed that the presence of a 32-kDa chitinase produced by normal plant cells allows embryo formation to occur (De Jong *et al.*, 1991). After studying several other compounds it was discovered that the *Rhizobium* lipo-oligosaccharides were also effective in rescuing the embryo mutants (De Jong *et al.*, 1993). These findings have led to the hypothesis that a chitinase produced by the carrot cells is responsible for releasing a signal molecule analogous to the *Rhizobium* Nod factors from a larger and so far unidentified N-acetylglucosamine-containing precursor, present in low amounts in the plant cell wall, which would allow for the formation of embryos. During the past few

years a number of research groups have started to examine the different effects chitinases may have on the function of the *Rhizobium* Nod factors. For example, it has been demonstrated that by using lipo-oligosaccharide preparations from *Rhizobium* and *Bradyrhizobium* root chitinases purified from different legumes have the capability to inactivate the Nod factors and prevent nodule formation (Staehelin *et al.*, 1992; Staehelin *et al.*, 1994). On the other hand, the *chiA* gene of *S. marcescens* has been transferred to *Rhizobium meliloti* and it has been demonstrated that expression of this gene during symbiosis on alfalfa roots does not interfere with nodule formation, but rather protects the plant against plant-pathogenic fungi (Sitrit *et al.*, 1993).

If *A. tumefaciens* does require a signal system similar to the Nod signaling system of *Rhizobium* in order to be oncogenic then one question that must be addressed is why does it appear that of all the bacterial and plant chitinase genes studied only the *chiB* gene of *S. liquefaciens* has the ability to inactivate the signal. A comparison of the nucleotide and amino acid sequences of the *S. liquefaciens chiB* gene with other plant and bacterial genes, that have successfully been transferred to plants, has revealed that there is only weak homology between the *chiB* gene of *S. liquefaciens* and the *chiA* gene of *S. marcescens* (Woytowich, 1991). For example, an examination of an 81% amino acid overlap of the two polypeptides showed that there was only 32.1% similarity. The homology of the two polypeptides began at the amino acid 339 of the ChiB polypeptide and the amino acid 474 of the ChiA polypeptide. Since ChiB does not share strong homology to other chitinases that have been transferred to plants it may be that the activity of this particular polypeptide is so much greater than that of other chitinases

studied that it destroys all or most of the "Nod-like" signals required for tumor and callus formation.

Since it appears that some type of chitin-like signal may be involved in callus and tumor formation by *A. tumefaciens* it was of interest to determine if the expression of *chiB* is critical for inactivating this signal within the first few hours of the *Agrobacterium*-mediated transformation or if its expression plays a role 18-24 hours after infection. From the chitinase assays undertaken in this study it became apparent that when *chiB* is under the control of the 35S-35S CaMV promoter it produces high levels of chitinase within the host bacteria. Since the tobacco discs used in each transformation were placed on co-cultivation plates for three days following infection it is very likely that the replicating *A. tumefaciens* cells continued to produce these high levels of chitinase. In order to determine if there is a specific time when the proposed signals are most affected by the presence of *chiB*, wounds were inflicted on the stems of *K. blossfeldiana* plants and then inoculated with the wildtype *A. tumefaciens* strains 348 and 281. Approximately 18 hours after the wounds had been inoculated with the wild type strains cultures of GV3101[pAW446] and GV3101[pHS723] were applied to the wound sites. Within four weeks of application of the *A. tumefaciens* strains carrying pAW446 and pHS723, galls started to form at the wound sites originally infected with *A. tumefaciens* 348 and 281 and then GV3101[pHS723]. However, galls did not form at the wound sites where GV3101[pAW446] had been applied 18 hours after initially being inoculated with the wild type *A. tumefaciens* strains. The findings from this set of experiments suggest that the "signal" proposed to have a role in the transfer of T-DNA to

the plant cells and the formation of galls does not necessarily occur immediately upon infection, but after a period of at least 18-24 hours following infection.

Since it has been shown that the *nodC* gene of *Rhizobium* is responsible for the production of chitin-oligosaccharides in the *Rhizobium*-plant symbiosis reaction it was of interest to determine if *A. tumefaciens* contains a gene that is homologous to *nodC* (Debelle *et al.*, 1992; Geremia *et al.*, 1994; Barny and Downie, 1996) . By using Southern blot analysis DNA extracted from *A. tumefaciens* C58 was probed with an 800-bp fragment of the *nodC* gene of *Rhizobium meliloti,* but no bands appeared on the autoradiogram. These results indicate that *A. tumefaciens* C58 does not have a gene that shares significant homology to the specific portion of *R. meliloti nodC* which was used as a probe. It is possible that a gene with a similar function as *nodC* is present in *A. tumefaciens* but that its nucleotide sequence is not homologous to that of the nodC genes isolated from different *Rhizobium* species. Some research groups have isolated *A. tumefaciens* genes that appear to be involved in tumorigenicity but the roles of only some of them have been defined. For example, transcript 6b, also known as *tml,* and transcript 5 have been found not to be oncogenic alone, but appear to modulate tumorigenicity when present in combination with other oncogenes (Spanier *et al.*, 1989; Tinland *et al.*, 1989). Unfortunately, the exact roles these genes play in tumor formation have been poorly characterized. Hence, genes that have been isolated from *A. tumefaciens* and appear to be involved in tumor formation may actually be responsible for playing a role in producing or interacting with the "nod-like" signal molecule that has been proposed in this dissertation.

To prove the hypothesis put forth in this thesis that the presence of chitin-oligosaccharides, which are probably structurally similar to the Nod factors of *Rhizobium* spp., are necessary for *A. tumefaciens* to be oncogenic several more studies would have to be undertaken. Such studies could entail preparing genomic libraries from strains of *A. tumefaciens* such as C58, 348 and 281 or others and screening for genes which show homology to the different *nod* genes of Rhizobia especially the different *nodC* genes and that have been isolated and sequenced. It would also be of interest to check for the presence of compounds from *A. tumefaciens* that are structurally similar to *Rhizobium* nod factors. Finally, other bacterial or plant chitinase genes that have been isolated, but not transferred to plants could be examined to determine the exclusivity or inclusivity of observations made in this study.

3.4 Summary and conclusions

From the results reported in this portion of the dissertation it became evident that *Agrobacterium*-mediated transformation of tobacco and *B. napus,* by the strain *A. tumefaciens* GV3101, was inhibited by the presence of the *chiB* gene of *Serratia liquefaciens.* Further examination of this phenomenon revealed that gall formation around wound sites on the stems of tobacco, tomato and *Zinnia* plants was also inhibited by the presence of *chiB.* When the expression of *chiB* was inhibited by the introduction of deletion and frameshift mutations, the ability to form galls was restored. Additionally, *Agrobacterium*-mediated genetic transformation of tobacco with the mutated *chiB* genes was also found to be successful. In this study it was also found that *chiB*-containing *A. tumefaciens* has the ability to inhibit the formation of galls at wound sites that had been inoculated approximately 18 hours beforehand with the strain *A. tumefaciens* C58. However, when *chiB*-carrying plasmid was replaced in the strain *A. tumefaciens* C58[pAW446] with one lacking the gene the resultant strain was found to regain its oncogenic ability. From all of these experiments it is evident that the presence of the *chiB* sequence in different *A. tumefaciens* strains leads to the abolition of the strains' oncogenic ability. Thus, it is proposed in this thesis that the presence of *chiB* inhibits the tumorigenic activity of Agrobacteria by interfering with a signal.It is further speculated that this signal may be "chitinaceous" factors such as the Nod factors of *Rhizobium.* ChiB may interfere by degrading or modifying the signal.

4.0 GENETIC MANIPULATION OF A SYNTHETIC ANTIMICROBIAL PEPTIDE GENE IN TOBACCO AND CANOLA

4.1 Literature review

4.1.1 Defense responses in plants

When plants are challenged by potential pathogens a complex progression of biological and molecular interactions occurs that culminate in symptoms associated with disease or disease resistance. In some plants a series of dynamic defense mechanisms can also be triggered by wounding and to some degree by non-pathogenic organisms. The events that determine host resistance are limited to those cells that are within a narrow zone that defines the area of contact between the invading pathogen and the host (Graham and Graham, 1991). Generally, there are three major defense responses that occur in plants: (a) accumulation of structural host cell wall proteins; (b) accumulation within the host of antibiotics and cell wall-bound secondary metabolites; and (c) production of antimicrobial proteins.

Fortification of cell walls is one of the first defense mechanisms used by plants when pathogen structures, such as fungal hyphae, attempt to penetrate it. This is accomplished by depositing newly formed carbohydrate material in the cell wall (Aist, 1976) and by oxidatively cross-linking pre-existing cell wall proteins (Bradley *et al.*, 1992). The accumulations of the structural host cell wall proteins known as the

hydroxyproline-rich glycoproteins (HRGPs) play an important defensive role and have been well studied. There are basically two classes of HRGPs that are believed to be involved in the defense of plants against pathogens. The first class are the insoluble extensins and they are thought to play a major role in strengthening the cell wall. The second class includes the soluble extensins and the lectin-active HRGPs. These HRGPs are believed to be involved in pathogen immobilization due to the agglutination activity they display (Leach *et al.*, 1982; Bolwell, 1987).

O'Connell and co-workers (1990) have studied the immunocytochemical localization of extensin in bean and melon plants inoculated with several pathogens. In one case, the pathogen *Pseudomonas fluorescens* was found not to cause any visible symptoms on the leaves of either host. It was found that within six hours of inoculation extensin antibodies were bound to the paramural region associated with membrane invagination and with amorphous material surrounding the bacteria. From these results it was concluded that the early appearance of the HRGPs in the material encapsulating the bacteria is consistent with the involvement of these glycoproteins in agglutination and immobilization of the bacteria on the plant cell walls. When the pathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola* was studied, a hypersensitive response was observed in the leaves of both bean and melon. Examination of these leaves indicated that within three hours of inoculation extensin was localized in the paramural area.

Plants also respond to pathogen attack by producing secondary metabolites. These include preformed antibiotics, induced antibiotics known as phytoalexins, and covalently linked cell wall phenolics that act to strengthen plant cell walls (Van Etten *et al.*, 1989; Graham and Graham, 1991). The production of phytoalexins depends on the

transcriptional activation of a series of genes that encode the enzymes of the phytoalexin biosynthetic pathway. A number of products derived from several different pathways are known to play a role in resistance, but the most studied pathway is the phenylpropanoid pathway. This pathway is universally distributed and information is available on its metabolites. At the present time it appears that upon attack the plant cells activate the genes for early phenylpropanoid and flavonoid metabolism. Following this, the genes for phytoalexin accumulation are activated. It is possible that these events may first result in the deposition of wall-bound phenolics (Jahnen and Hahlbrook, 1988), followed by the hydrolysis of isoflavone conjugates (Graham *et al.*, 1990) and finally the accumulation of phytoalexins.

When confronted with a pathogen plants also produce a number of defense-oriented proteins, referred to as pathogenesis-related proteins, that play a direct role in the control of the microorganism. True pathogenesis-related proteins share certain attributes such as low molecular weight, resistance to proteolytic enzymes, extraction at low pH and presence in the apoplast of infected tissues (Van Loon, 1985). Several classes of defense-related proteins have been identified and they include enzymes involved in phenylpropanoid metabolism, hydrolytic enzymes such as chitinases and β-1,3-glucanases, and other proteins such as permatins, thionins, toxin detoxifying proteins, chitin-binding lectins and ribosome-inactivating proteins. There are also a number of other defense-related proteins that have recently been isolated whose exact mode of action is not known.

Small, highly basic and cysteine-rich proteins are also known to play a role in a host plant's defense. These proteins are widely distributed phylogenetically and they are

known to occur in invertebrates, vertebrates, fungi and plants (Lambert *et al.*, 1989; Lehrer *et al.*, 1993; Nakaya *et al.*, 1990; Bohlmann and Apel, 1991). Some of the plant proteins that appear to play a defensive role in plants include thionins, hevein-like proteins, defensins and antimicrobial peptides isolated from the seeds of *Raphanus sativus, Mirabilis jalapa* and *Arabidopsis thaliana*.

4.1.2 Antimicrobial peptides

A number of small peptides that display the ability to inhibit the growth of fungi and bacteria have been isolated from plants. Some of these peptides are approximately 50 to 100 amino acids in length and are classified into disulfide-linked peptides, linear peptides and lantibiotics (Bessalle *et al.*, 1993; Bohlmann and Apel, 1991; Boman *et al.*, 1991; Cammue *et al.*, 1992; Hansen, 1993; Zhong *et al.*, 1994). Recently, studies have been made in uncovering the structure-activity relationships of these peptides in hopes of being able to better control certain plant diseases.

4.1.2.1 Disulfide-linked peptides

The disulfide-linked antimicrobial peptides studied to date are complex molecules with internal disulfide bridges which dictate the conformation of the molecules for biological activity. There are several families of peptides that fall into this category such as insect and mammalian defensins (Hoffmann and Heru, 1992; Lehrer *et al.*, 1993), thionins (Bohlmann and Apel, 1991), brevenin (Morikawa *et al.*, 1992) and antimicrobial peptides from amaranth and *Mirabilis jalapa* (Broekart *et al.*, 1992; Cammue *et al.*, 1992).

Defensins have been isolated from a number of mammals such as humans, rats, rabbits and guinea pigs (Lehrer *et al.*, 1993). Generally, these proteins are highly cationic, consist of 30-35 residues with conserved cysteine residues and exhibit cytotoxicity against several types of mammalian cells. These specific proteins have also been demonstrated to have antimicrobial activity against numerous Gram-negative and Gram-positive bacteria, mycobacteria, spirochetes, and some human pathogenic fungi such as *Aspergillus fumigatus*. Defensins with conserved cysteine residues have also been isolated from different insects. These include such proteins as sapecin (Matsuyama and Natori, 1988) and phormicin (Lambert *et al.*, 1989). Recently, it has also been revealed that a synthetic, 11 residue hendecapeptide derived from an α-helical region of sapecin B has superior antimicrobial activity when compared to the parent peptide (Yamada and Natori, 1994). Since this peptide has the capability of releasing untrapped glucose from acidic liposomes it has been suggested that its microbial function may involve a lytic mechanism.

A very potent disulfide-linked antimicrobial peptide has been isolated from *Tachypleus tridentatus* (Horseshoe crab) (Nakamura *et al.*, 1988). This peptide, known as tachyplesin, is smaller than the defensin family of proteins. It is a hemolytic, cationic peptide that is only 17 residues long, contains two disulfide bonds and has been shown to exhibit activity against *Candida albicans*. Further studies undertaken on this particular peptide have suggested that its antimicrobial function is related to its amphipathic structure. This structure arises from the distribution of bulky hydrophobic groups at the head of the antiparallel β-pleated sheet structure which is linked via a turn to a

hydrophilic tail. The tail is comprised of six cationic residues that are derived from the N and C termini of the peptide (Ohta *et al.*, 1992; Katsu *et al.*, 1993).

Another interesting source of several linear and disulfide-linked antimicrobial peptides is the skin tissue of amphibians. Clark and co-workers (1994) examined a peptide, known as ranalexin, from the bullfrog *Rana catabeiana.* This cationic antimicrobial peptide has 20 amino acids and contains a single disulfide bond at the C terminus. Examination of a molecular model of ranalexin has indicated that its structure is similar to the membrane active antibiotic polymixin, a cyclic peptide. The similarity encompasses an amphipathic structure comprising a head region containing a cationic ring and a tail region made up of hydrophobic residues arranged in an α-helix. Two other *Rana* species also produce highly homologous antimicrobial peptides. These include the 24-residue brevinin-1 and 33-residue brevinin-2 proteins from the Japanese frog *Rana brevipoda porsa* and the brevinin-1E and brevinin-2E proteins from the European frog *Rana esculenta* (Morikawa *et al.*, 1992; Simmaco *et al.*, 1993). Another example of an antimicrobial peptide is esculentin which also occurs in the European species of the bullfrog.

Disulfide-containing antimicrobial peptides have also been isolated from several different plants, the most studied of which are probably the thionins. Thionins are a family of low-molecular weight proteins that have been found to be very toxic against bacteria, fungi, yeast and animal and plant cells (Bohlmann and Apel 1991, Molina *et al.*, 1993). Thionins have been isolated from several different species of plants such as wheat, barley, oat, maize, rye and mistletoe (Fernandez de Caleya *et al.*, 1976; Ozaki *et al.*, 1980; Bekes and Lasztity, 1981; Jones and Cooper, 1980; Hernandez-Lucas *et al.*,

1978; Samuelsson, 1958). All the thionins studied to date contain 6-8 cysteine residues. In addition to the homology present in the primary structure, thionins also share homology in their three dimensional structure. All the thionins studied display a three dimensional structure in which they are compact, L-shaped polypeptides where the long arm of the L is formed by two α-helixes. The short arm is formed by two short antiparallel β-sheets and the last C-terminal amino acids (Bohlmann and Apel, 1991). This three dimensional structure is then stabilized by three or four disulfide bridges. Finally, the biological activity of these peptides appears to be associated with a membrane-lytic function.

Several small, cationic antimicrobial polypeptides that do not fall into the thionin family of proteins have also been isolated from different plants. Duvick and co-workers (1992) have isolated such polypeptides from the kernels of corn. One of the peptides, referred to as MBP-1, is a 33 amino acid α-helical protein that is rich in arginine and contains two disulfide bridges. This peptide is active against a number of fungal pathogens of maize and also exhibits antibacterial activity. Peptides of similar size have also been isolated and characterized from the seeds of *Amaranthus caudatus* (Broekaert *et al.,* 1992). These peptides, termed *Ac*-AMP1 and *Ac*-AMP2, are highly basic and contain three disulfide bridges. They are toxic to six species of plant-pathogenic fungi and Gram-positive bacteria. The seeds of *M. jalapa* have also been found to be the source of two antimicrobial peptides known as *Mj*-AMP1 and *Mj*-AMP2 (Cammue *et al.,* 1992). These two peptides consist of 37 and 36 residues, respectively. They are also toxic against several plant pathogens as well as Gram-positive bacteria. More recently two small cysteine-rich antifungal proteins, Rs-AFR1 and Rs-AFR2 which are 5-kDa and

shown to exert antifungal activity against a broad spectrum of plant pathogenic filamentous fungi, have been characterized from radish seeds (Terras *et al.*, 1995). These peptides function by causing hyperbranching and growth reduction of the fungal hyphal tips. However, they have little effect on bacteria or cultured human cells.

4.1.2.2 Linear antimicrobial peptides

Several linear polypeptides with antimicrobial activity have been isolated from different organisms. Some examples of these polypeptides include cecropins from mammals and insects (Boman *et al.*, 1991), histatins from human parotid and submandibular secretions (Oppenheim *et al.*, 1988), dermaseptin from *Phyllomedusa sauvagii* (Mor and Nicholas, 1994), melittin and melittin analogs from bee venom (Blondelle and Houghten, 1991), drosocin from *Drosophila* (Bulet *et al.*, 1993) and antimicrobial peptides from bovine neutrophils (Frank *et al.*, 1990).

Cecropins are a family of homologous peptides of 35-37 residues (Boman *et al.*, 1991). It is believed that the antibacterial activity of these polypeptides is linked to a molecular architecture consisting of a cationic N-terminal amphipathic region and a hydrophobic C-terminal amphipathic half separated by a hinge. Fink and co-workers (1989) have confirmed the role of the structure of the peptide in relation to its biological activity by designing and synthesizing several analogs. The cecropins affect prokaryotic cells, but they do not have an effect on eukaryotic cells. Research undertaken on the design and synthesis of cecropinlike peptides has been very successful. For example, Shiva-1, a synthetic peptide with approximately 46% homology to cecropin, has been shown to have superior biological activity (Jaynes, 1990). As well, a synthetic D-isomer

of cecropin B has been found to retain full biological activity (Wade *et al.*, 1990). Since both natural and synthetic cecropin polypeptides possess potent antimicrobial activity, but do not adversely affect the plant cells, it is very likely that these polypeptides will be useful in potential disease resistance strategies.

There are also other insects that secrete linear antibacterial peptides. One such example is the bee venom peptide known as melittin. This peptide is structurally similar to the cecropins, but contains the undesirable characteristics of being toxic and hemolytic. In order to eliminate the hemolytic property, the residues essential for this function have been mapped and several cecropin-mellittin hybrids synthesized (Blondelle and Houghten, 1991). These hybrids no longer possess the hemolytic function, but they retain superior antibacterial activity.

Apidaecins are a family of heat-stable, proline-rich peptides that are produced by the honeybee (*Apis mellifera*). These peptides consist of approximately 18 amino acids and show marked activity against many plant-associated bacteria by means of a non-lytic mechanism (Casteels *et al.*, 1993). It is this non-lytic mechanism that makes the apidaecins very attractive candidates for possible plant defense proteins.

There are some antimicrobial linear peptides that have been isolated from humans that may have the potential to play a defensive role in plants. Histatins are a class of antimicrobial peptides that vary from 7-38 amino acids and are rich in histidine (Oppenheim *et al.*, 1988). They were isolated from the parotid and submandibular secretions of humans and have been determined to be active against a number of infectious microorganisms present in the oral cavity. They also display very potent fungistatic effects on *C. albicans*. Some of the shortest linear antimicrobial peptides

isolated were derived from human neutrophil lysosomal Cathespin G (Shafer *et al.*, 1991). These peptides contain the sequence Ile-Ile-Gly-Gly-Arg and His-Pro-Gln-Tyr-Asn-Gln-Arg and are broad spectrum antibiotics.

Several amidated, non-hemolytic prokaryote-specific peptides possessing antimicrobial activity have been isolated from skin secretions of *Xenopus laevis* (Gibson *et al.*, 1991). These skin secretions also contain the peptide magainin (Zasloff, 1987). This magainin family of antimicrobial peptides has also been isolated from the stomach of this particular species of frog (Moore *et al.*, 1991). Although the precise mechanism of the antimicrobial action of magainin is not fully understood, it is effective against a variety of protozoa, bacteria and fungi, but not against eukaryotic membranes. Ludtke *et al.* (1994) found that the ability of these peptides to adopt an amphipathic α-helical structure in the presence of phospholipid bilayers plays an important role in its autolytic activity. Dermaseptin is another amphibian antimicrobial peptide that was isolated from the skin secretion of the South American arboreal frog *Phyllomedusa sauvagii* (Mor *et al.*, 1991). Dermaseptin is not related to the maganins. It is a 34 amino acid non-hemolytic peptide in which the amino-terminal amphipathic α-helical domain, which comprises 18 residues, is responsible for its potent antimicrobial activity (Mor and Nicolas, 1994).

4.1.2.3 Lantibiotics

Several Gram-positive bacteria have been determined to produce polypeptides, termed bacteriocins, with bactericidal activity against related species. Lantibiotics are another class of antimicrobial peptides that are less than 4 kDa (Schnell *et al.*, 1988).

These peptides undergo a posttranslational modification that leads to the creation of unusual amino acids such as dehydrobutyrine and dehydroalanine. These amino acids then condense with neighboring cysteine residues to give rise to lanthione and β-methyllanthionine which are thioether amino acids. Probably the three best known lantibiotics are epidermin, minisin and subtilin. All three of these polypeptides are active against many Gram-positive bacteria, but there is no information on the effect they may have on specific plant pathogens.

4.1.2.4 Synthetic antimicrobial peptides

The cationic nature of all the different classes of antimicrobial peptides isolated and characterized and their ability to form an amphipathic secondary structure upon interaction with the surface of the cell membrane leads to the formation of ion channels which results in cell lysis and death of the pathogenic organism. These two properties have led to the design and synthesis of new peptides with antimicrobial activity. Several researchers have been successful in synthesizing these types of peptides. Blondelle and Houghten (1992), for example, successfully synthesized amphipathic peptides that contain only redundant leucine and lysine. They were able to show that antimicrobial activity could be removed from the concomitant hemolytic activity by certain nucleotide sequence deletions or substitutions. As well, several model peptides ranging from 9-17 amino acids have been designed (Bessalle *et al.*, 1993) and upon testing it was found that some of the smaller peptides, in the absence of an amphipathic helical structure, have high levels of antimicrobial activity. More recently, putative cationic amphipathic structures of such protein sequences have been identified and engineered to display a

broad spectrum of antibacterial activity as well as activity against two maize fungal pathogens (Zhong *et al.*, 1994).

4.1.3 Antimicrobial peptides of *Mirabilis jalapa* L.

A class of potent antimicrobial peptides has been purified from the seeds of the four o'clock plant (*M. jalapa* L.) (Cammue *et al.*, 1992). These proteins, designated AMP1 and AMP2, are highly basic and consist of 37 and 36 residues respectively. These proteins contain three disulfide bridges and only differ from each other by four amino acids. The antifungal potency of AMP1 and AMP2 has been examined using 15 different plant pathogenic fungi (Cammue *et al.*, 1992). The concentrations required for 50% inhibition of fungal growth varied from 6 to 300 μg/mL for AMP1 and 0.5 to 20 μg/mL for AMP2. These two peptides were also tested against Gram-negative and Gram-positive bacteria and they were found to be active against *Bacillus megaterium* and *Sarcina lutea*, but not the Gram-negative bacteria tested. Both proteins show sequence homology to μ-agatoxins, a class of insecticidal neurotoxic peptides isolated from the venom of spiders. However, AMP1 and AMP2 do not affect nerve impulse transmission in insects.

Two cDNA clones, designated MJ1 and MJ2, which encode the AMP1 and AMP2 peptides have recently been isolated and characterized (DeBolle *et al.*, 1995). Examination of the deduced amino acid sequences of both proteins revealed the presence of a putative signal sequence, which suggests that the AMPs are expressed as preproteins. The MJ1 and MJ2 genes both contain a single open reading frame and Southern blot analysis has revealed that these genes belong to a gene family of low complexity. DeBolle and co-workers (1995) also examined the spatial expression pattern of these

genes using northern blot analysis. These experiments revealed that transcripts of the expected size could only be detected in seeds and not in roots, flowers or leaves. Furthermore, it was found that mRNA starts to accumulate in seeds at the onset of desiccation reaching a maximum level in dry, mature seeds. To determine if the genes may be expressed in leaves by stress, leaves were subjected to mechanical wounding, infection by *Botrytis cinerea* and the application of salicylic acid, but the results of DeBolle and co-workers (1995) revealed that the expression of the AMP genes is restricted to the seeds even under various forms of stress.

4.1.4 Possible use or application of antimicrobial peptides in plant biotechnology

Research on small antimicrobial peptides has shown that these peptides may be useful for developing important crop plants that have a superior ability to resist diseases caused by a wide variety of plant pathogens. For this to occur it is necessary that the crop of interest be easily transformed with the gene encoding the desired peptide. Currently, reports regarding the expression of peptides in plants are limited. Carmona and co-workers (1993) found that tobacco plants expressing the barley α-thionin have an enhanced resistance to bacterial pathogens. As well, proteins extracted from potato plants expressing the cecropin gene also show antibacterial activity (Montanelli and Nascari, 1991). When Shiva-1, the modified cecropin B peptide, was expressed in tobacco, enhanced resistance to *Pseudomonas solanacearum* was observed (Jaynes *et al.*, 1993). Recently, Terras and co-workers (1995) successfully transferred the *Raphanus sativus*-antifungal protein 2 (Rs-AP2) gene into tobacco and found that the transgenic plants displayed enhanced resistance to the foliar pathogen *Alternaria longipes*. Furthermore,

preliminary results obtained from tissue printing of diseased leaves have shown that the amount of Rs-AFP accumulating locally around infection sites is higher than that in the remaining part of the leaf. From results such as these it seems possible that the introduction of natural or synthetic antimicrobial peptide genes, alone or in combination, through plant biotechnology may play a key role in enhancing resistance of plants to invading pathogens.

4.2 Results

4.2.1 Genetic manipulation of the *amp2* gene

At the Third International Society for Plant Molecular Biology meeting, in October 1991, data were presented on the isolation and characterization of two proteins with antimicrobial activity from *M. jalapa*. The amino acid sequences of these proteins, AMP1 and AMP2, were used to derive the coding sequences according to the most common codon usage found in a number of different dicots, including *B. napus*. Since subsequently Cammue *et al.* (1992) indicated that antimicrobial activity of AMP2 is much greater than AMP1, it was of interest to focus attention on AMP2 (Fig. 4.1)

A synthetic *amp2* gene was assembled by the candidate from two oligonucleotides which consisted of approximately 15-bp that are complementary to one another (Fig. 4.1). The two oligonucleotides were annealed to one another and filled-in using Pol KI and dNTPs. The resulting gene was amplified by PCR, passed through a Qiagen PCR clean up system, digested with *Nco* I and *Bam* HI and cloned into the expression vector pSE380 resulting in the clone pAW70 (Fig. 4.2). The nucleotide sequence of the synthetic gene was determined to ensure that no nucleotides had been missed or altered. The gene was then cloned into a plant transformation vector via suitable intermediate vectors.

1
(a) **M V C I G N G G R C N E N V G P P Y C C S G F C L R Q P N Q G Y G**
37
V C K N

1 25
(b) **ATG GTG TGC ATT GGA AAC GGT GGA AGA TGC AAC GAG AAC**
50 75
GTT GGT CCA CCA TAC TGT TGC TCT GGA TTC TGC CTT AGG
100
CAA CCA AAC CAA GGA TAC GGT GTT TGC AGG AAC AGA
117
TAG

Figure 4.1. Amino acid sequence of AMP2 (Cammue *et al.*, 1992) (a) and nucleotide sequence (b) of the synthetic *amp2* gene used in this study. The amino acids underlined were added to the published sequence when the synthetic DNA was designed for cloning into desired vectors.

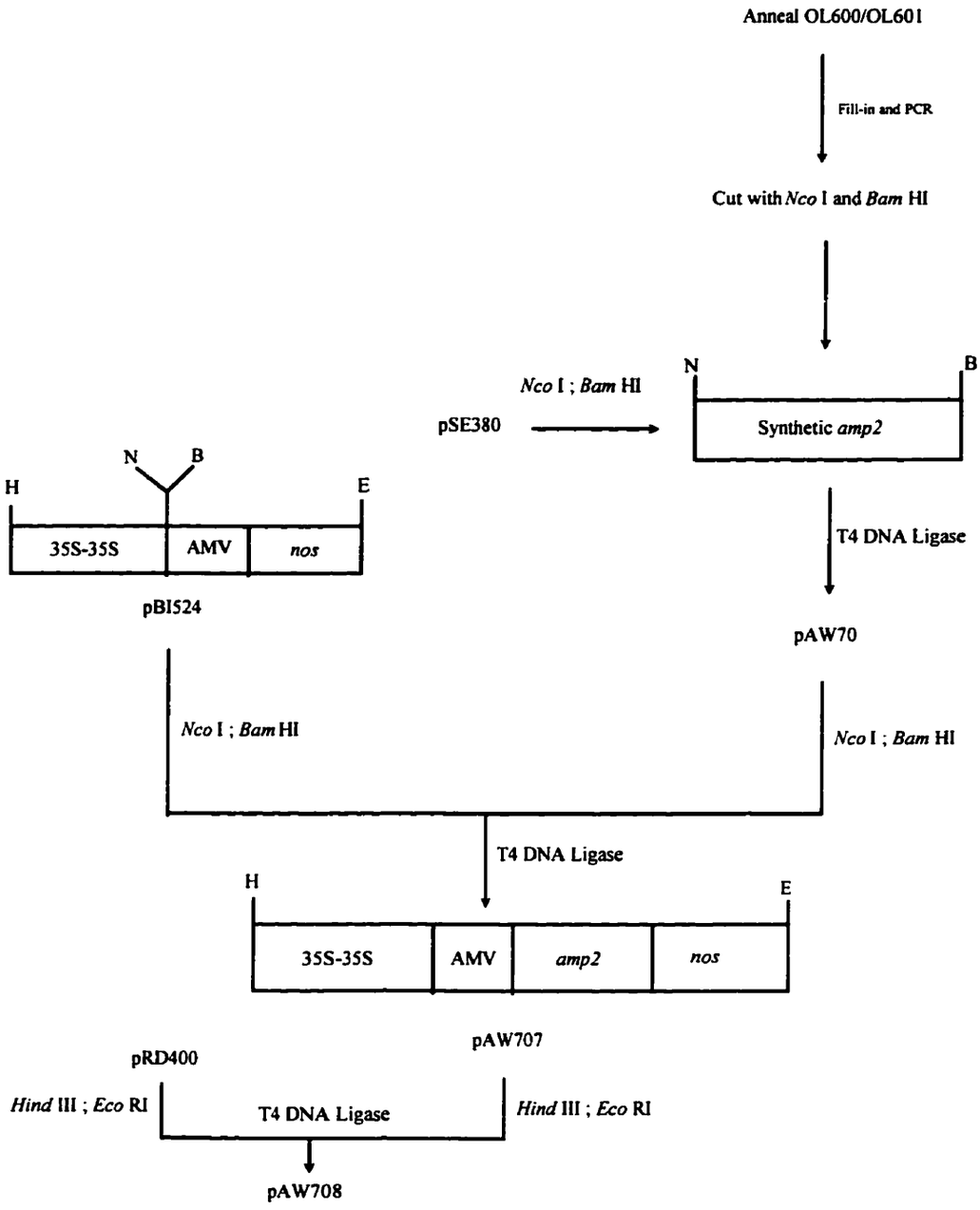

Figure 4.2.Construction of the *amp2* expression cassette and vector. 35S-35S, partially duplicated 35S CaMV promoter; AMV, alfalfa mosaic virus enhancer; *nos*, nopaline synthase terminator; *amp2*, antimicrobial peptide gene. Key restriction enzyme abbreviations: B, *Bam* HI; E, *Eco* RI; H, *Hind* III; N, *Nco* I.

4.2.2. Biological activity of the *amp2* gene product

Since many of the subsequent genetic manipulations in this study required the use of the strain *E. coli* DH5α it was necessary to determine if the AMP2 protein is toxic to the *E.coli* cells from within and from outside. DH5α [pAW70] was allowed to grow for 18 hours at 37°C in the presence of IPTG to induce *amp2* expression and protein extracts from the IPTG-induced and a non-induced control culture were tested against *E. coli* DH5α. Since IPTG induction did not affect growth of the strain and the extracts did not inhibit DH5α growth, it was concluded that the AMP2 protein is not toxic to the *E. coli* strain DH5α.

When the synthetic *amp2* gene was sequenced it was found to be complete as no bases had been altered. However, before any further manipulations were undertaken the *amp2* gene product was tested to determine if it was biologically active. The test organism used to determine the biological activity of the *amp2* gene product was *B. megaterium*. Cammue *et al.* (1992) had previously demonstrated that AMP2 protein isolated from the seeds of *M. jalapa* strongly inhibited the growth of the Gram-positive bacterium *B. megaterium*. In order to assay the biological activity of the synthetic *amp2* gene, protein extracts were prepared from the *E.coli* strains DH5α, DH5α [pSE380] and DH5α [pAW70]. The protein extracts were spotted on sterile Whatman 3M discs which were placed onto a 2YT plate which had been overlayed with top agar containing *B. megaterium*.

The plates were incubated at 30°C for 18 hours after which they were examined for the presence of a clearance or halo around the discs that would indicate the inhibition of

bacterial growth. As illustrated in Fig. 4.3, a 3-mm zone of inhibition was observed around the disc which contained the protein extract from DH5α[pAW70]. No inhibition of growth, however, was observed around the two control discs containing extracts from DH5α and DH5α [pSE380]. This experiment was repeated twice and the same results were obtained each time. It was concluded, therefore, that the gene product of the synthetic *amp2* is biologically active against *B. megaterium.*

4.2.3. Introduction of the synthetic *amp2* gene into tobacco

The synthetic *amp2* gene was subcloned from pAW70 as an *Nco* I-*Bam* HI fragment into the vector pBI524-14 containing a plant expression cassette to yield the plasmid pAW707. The nucleotide sequence of the gene and the flanking ends of the 35S-35S promoter and *nos* terminator were then determined. The cassette was cloned into the plant vector pRD400 (Datla *et al.*, 1992) as a *Hind* III to *Eco* RI fragment (Fig. 4.2) and the resulting clone, pAW708, was transferred to *A. tumefaciens* strain GV3101 by the freeze-thaw method. Plasmid DNA from the resulting colonies was restriction digested to ensure integrity of the plasmid. The *A. tumefaciens* culture was then used in the genetic transformation of tobacco. The tobacco was transformed by means of the leaf disc transformation-regeneration method of Horsch *et al.* (1985). Following infection of 20 discs 18 plants were regenerated and all were tested for the integration of the *amp2* gene into the genome.

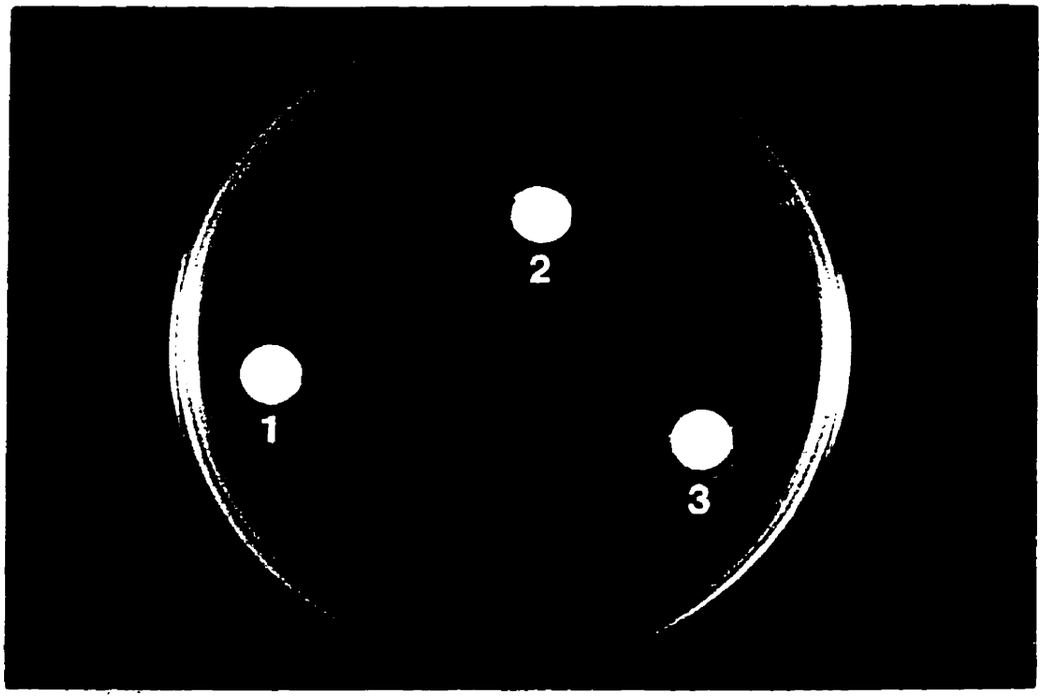

Figure 4.3. Plate assay to test for the production of antibacterial activity by the *amp2* gene product. Bacterial extracts (25 μg of protein) were spotted on plates seeded with *B. megaterium*. Discs 1, 2 and 3 contained proteins extracted from DH5α, DH5α[pAW70] and DH5α[pSE380], respectively.

4.2.4. Identification of *amp2* in regenerated tobacco plants

From the leaf disc transformation with pAW708 a total of 18 plants (numbered as AW708-1 through 18) were regenerated and transferred to potting soil. Two of the plants, AW708-10 and AW708-17, did not survive once transferred to potting soil and therefore, they were excluded from this study. Genomic DNA was extracted from the remaining 16 plants, as well as the parental wild type tobacco plant. PCR was then used to identify the presence of *amp2* by using two oligonucleotides specific for the 5′ and the 3′ end of the synthesized gene. If *amp2* is present in the genome of the particular plant being tested then amplification of a 118-bp fragment should occur. This 118-bp fragment was present in all the transgenic plants tested except for pAW708-13. As expected, the 118-bp fragment was absent in the sample containing DNA extracted from the wild type plant and it was concluded that *amp2* integrated into the genomes of 15 out of the 16 regenerated plants.

Further confirmation of the presence of *amp2* was obtained using the TA cloning system produced by InVitrogen. The TA cloning system is a plasmid cloning kit designed specifically for PCR products. It takes advantage of the non-template dependent activity of thermostable polymerases used in PCR that add a single deoxyadenosine to the 3'-ends of duplex molecules. These can be cloned into vectors with a complementary, protruding "T"-end. The resulting clones can then be analyzed by restriction endonucleases and sequenced using the M13 reverse primer and/or the T7 promoter primer. Due to the number of plants to be screened, and the cost of the kit, it was not feasible to test every plant so PCR products from arbitrarily chosen plants were cloned into the pCRII plasmid provided in the kit, screened and sequenced. Amplified DNA from plants AW708-1,

AW708-4 and AW708-5 was ligated with pCRII and the resulting colonies screened with restriction enzymes. DNA was extracted from colonies which displayed the restriction digest banding pattern and sequenced. All three of these tobacco plants were positive for the presence of the *amp2* sequence. This led to the conclusion that at least in the select transgenic plants, whose DNA amplified a 118-bp PCR product, were positive for the presence of the synthetic *amp2* gene.

4.2.5 Progeny analysis of the transgenic tobacco plants

The *amp2* construct used for transformation contains a *npt*II expression cassette which is closely linked to the *amp2* expression cassette. The two cassettes are only separated by a distance of approximately 0.7 kb and therefore it is likely that these two genes will be inherited together. Thus, the segregation of the *npt*II gene can be taken as an indication of the segregation of the *amp2* gene.

The inheritance of *npt*II was followed by the segregation of resistant or sensitive F_1 tobacco seedlings, which arose from the seeds of selfed primary transformants (T_0), on 1/2 strength MS medium. As illustrated in Table 4.1, the test results clearly indicate that probably all of the transgenic plants contained one *npt*II locus which resulted in the segregation ratio of 3 resistant:1 sensitive plant. If two independent *npt*II loci had been present then a segregation ratio of 15 resistant:1 sensitive would have been observed. A chi-square test was performed to compare the observed frequencies of kanamycin resistant and sensitive seedlings with the test ratio.

Table 4.1 Test of significance of segregation of kanamycin resistance in the progeny of primary transgenic tobacco plants carrying the *amp2* sequence

Plant	Number of seedlings		Test ratio	χ^2 Value*
	green	white		
708-1	79	21	3:1	0.85
708-2	101	37	3:1	0.24
708-3	77	23	3:1	0.21
708-4	97	34	3:1	0.07
708-5	127	47	3:1	0.37
708-6	219	81	3:1	0.64
708-7	111	44	3:1	0.95
708-8	92	29	3:1	0.07
708-9	102	40	3:1	0.76
708-11	94	31	3:1	0.00
708-12	101	52	3:1	6.59
708-14	100	34	3:1	0.01
708-15	66	33	3:1	3.84
708-16	75	22	3:1	0.28

*Estimated χ^2 is not significant at the 5% level.

4.2.6 Bacterial assays of proteins isolated from transgenic tobacco plants

Preliminary assays were carried out, in duplicate, with protein extracts from all of the primary transgenic tobacco plants to determine if there they had an inhibitory effect on the growth of *B. megaterium*. Of the 15 primary transgenic plants tested only one appeared to have very little inhibition of bacterial growth. The tobacco plant AW708-1 showed a small zone of inhibition of approximately 1-2 mm outside the filter. Although the results obtained from the primary transgenic plants are not conclusive as a number of changes can occur to the plants as they undergo tissue culture, it is important to carry out studies on the progeny of primary transgenics. It should also be mentioned that it is not known until the F_2 generation if a plant of interest is homozygous or heterozygous for the gene being studied. Therefore, it is necessary to screen the T_0 and F_1 progeny seeds to see which plants are homozygous for *amp2*. It is with these tobacco plants that further tests have been undertaken.

Protein extracts from the homozygous plants and some of the heterozygous plants were assayed against *B. megaterium*, but none of them was found to inhibit the growth of *B. megaterium*. Since several previous studies (Rao, 1995) have shown that the activity of several antimicrobial peptides is inhibited by the presence of cations, crude protein extracts were passed through desalting columns and then assayed against *B. megaterium*. The majority of the desalted protein extracts assayed were also found not to have an effect, therefore suggesting that other factors may be interfering with the antimicrobial activity of AMP2. However, as illustrated in Figure 4.4 desalted protein extracts from the tobacco line AW708-1-1 did show very low levels of antibacterial

activity compared to non-transformed tobacco protein extracts when protein concentrations as high as 3 mg were assayed. The presence of the AMP2 protein within this extract was then confirmed by running proteins extracted from untransformed tobacco plants and AW708-1-1 plants on a SDS-polyacrylamide gel. Due to the small size of the AMP2 protein it is very difficult to observe the protein. However, extracts isolated from tobacco plants carrying the *amp2* gene sequence appear to produce a protein in the size range of 3-6 kDa that is not present in the extract from untransformed plants. (Fig. 4.5).

4.2.7 Screening tobacco plants for increased resistance to *R. solani* AG-1-1

To determine if the transgenic tobacco plants with the *amp2* gene were more resistant to the fungal pathogen *R. solani* AG-1-1 a series of assays were performed. These assays were undertaken by sowing tobacco seeds in sterile soil and two weeks later six seedlings from each plant line were transplanted to pots containing sterile soil and two grains of inoculated rye. The seedlings were then monitored daily for signs of growth which would indicate resistance to the pathogen. In all 14 lines, the seedlings never grew beyond the two leaf stage and died within five days of being exposed to *R. solani.* As expected, none of the wild type tobacco seedlings survived either. These results indicate

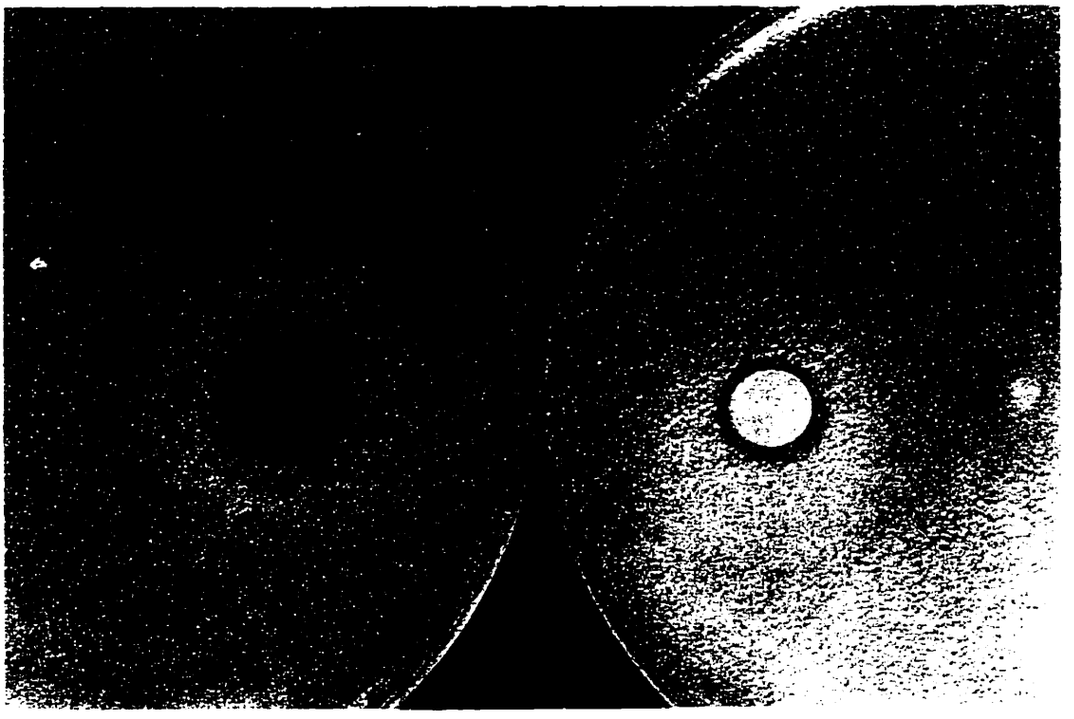

Figure 4.4. Plate assay to test for the presence of antibacterial activity in protein extracts isolated from transgenic *amp2* and untransformed tobacco plants. Plant extracts (3 mg/µl) were spotted on plates seeded with *B. megaterium*. Discs 1 and 2 contained proteins extracted from tobacco plants AW708-1-1-9 and wildtype, respectively.

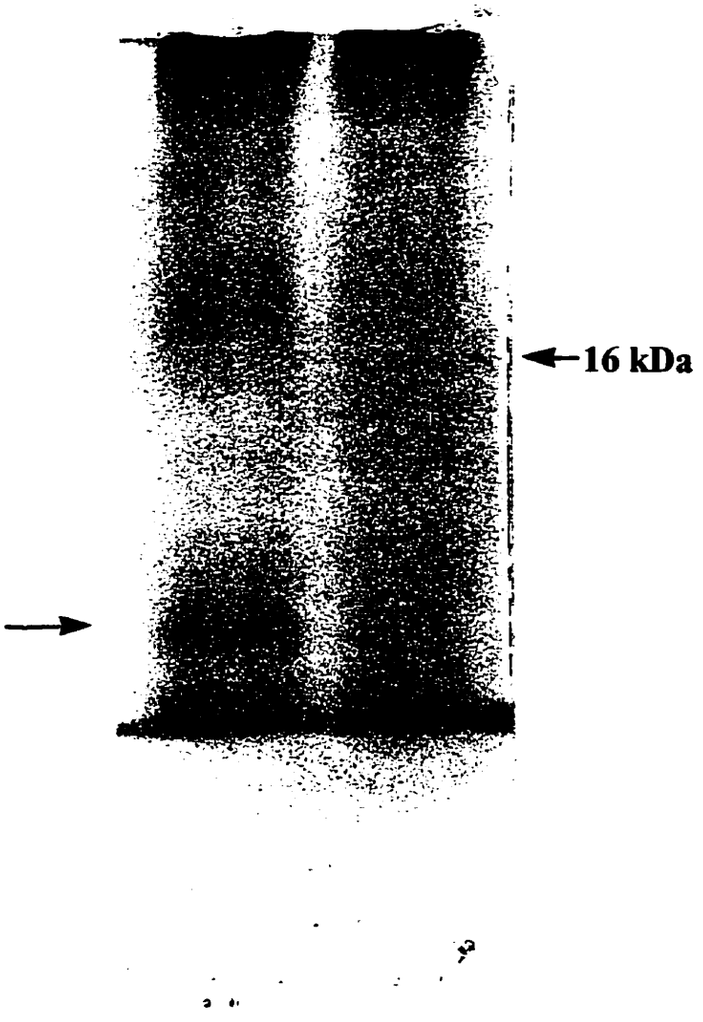

Figure 4.5. SDS-PAGE of desalted protein extracts isolated from AW708-1-1 (lane 1) and untransformed tobacco plants (lane 2) stained with Coomassie brilliant blue. A total of 100μg/50μl of extract was loaded into each well.

that tobacco plants transformed with the synthetic *amp2* gene do not show an increased level of resistance to the fungal plant-pathogen *R. solani* AG-1-1.

4.2.8 Introduction of the synthetic *amp2* gene into *B. napus* and identification of putative transgenics

Brassica napus (Westar) cotyledons were infected with the *Agrobacterium* strain GV3101[pAW708] using the cotyledonary transformation method. Over 600 cotyledons were infected and six plants were regenerated. The six plants from this transformation were found to be positive for the presence of the 118-bp fragment of the *amp2* gene in a PCR assay. However, amplification of this fragment was not observed in a reaction containing DNA from untransformed *B. napus*. In this portion of the study the TA cloning system was also used to further confirm the presence of *amp2* in two randomly chosen PCR. Upon sequencing the resulting colonies the *amp2* sequence was identified in plants AW708-1 and AW708-6.

Preliminary assays were undertaken with protein extracts from the primary transgenic canola plants to determine if there was an inhibitory effect on the growth of *B. megaterium*. Assays were done with proteins extracted from the leaves of plants pAW708-1, pAW708-2, pAW708-3 and wild type canola. None of the protein extracts appeared to have an inhibitory effect on the growth of *B. megaterium* although the bacterial cells around the disc containing the AW708-1 proteins appeared to be slightly inhibited. Each protein extract was also passed through a desalting column, but the same negative results were observed. Since the *amp2* did not display antimicrobial activity in

the canola plants except for possibly pAW 708-1, only this plant was used for more in-depth studies.

In *B. napus* it is not as easy as in tobacco to determine which F_1 offspring contains *amp2* in its genome because kanamycin selection is very leaky in germinating seeds. For this reason PCR of the F_1 progeny was employed to determine if the *amp2* gene was segregating as expected. Since plant AW708-1 seemed to be the most promising, seeds were plated on 1/2 MS and 9 out of 12 germinated. When these plants were tested for the presence of *amp2* by PCR it was found that of the 9 F_1 seedlings, serially labelled as AW708-1-1 through 9, AW708-1-2, -4, and -5 were negative for PCR amplification while the others were positive. As expected, no amplification of the *amp2* gene was observed in the reaction containing DNA from wild type *B. napus*. These results gave the expected 3:1 ratio if there was one-locus insertion with an estimated χ^2 value of 0.33. It was concluded, therefore, that the *amp2* gene was segregating normally in these transgenic canola plants.

Proteins extracts from the six PCR positive F_1 plants, were tested for growth inhibition on *B. megaterium* and found to be negative. The extracts were also passed through desalting columns, re-tested against *B. megaterium* and found not to inhibit growth. These assay results indicate that as in the case of tobacco the presence of the synthetic *amp2* gene protein extracts do not exhibit antibacterial activity.

4.2.9 Screening *B. napus* plants for increased resistance to *R. solani* AG-2-1

The results of the experiments just described indicate that although the synthetic AMP2 protein possesses antibacterial activity *in vitro,* upon becoming integrated into the

plant's genome antibacterial activity appears to be no longer observed. It is possible that this may have been due to the low levels of the protein or due to the protein being modified or degraded. Because of the importance of trying to develop canola with an increased tolerance to plant pathogens a final series of fungal assays were performed to determine if canola plants carrying the *amp2* gene show an increase in resistance when challenged specifically with a fungal pathogen. The pathogen used in this study was *Rhizoctonia solani* AG-2-1 because it is the main organism that is responsible for pre-emergence damping-off and root rot of both seedlings and adult canola plants, and it is a very common soil-borne pathogen in western Canada.

In this experiment seeds from seven F_1 transgenic canola plants, as well as wild type, were placed on lightly packed uninfected soil-free growth media and covered with infested soil-free growth medium containing approximately 100 viable particles of *R. solani* AG-2-1 infested rye grain. Four replicate pots were used for each seed variety tested. The criteria used to evaluate the disease reaction of these varieties were the percent emergence after one week and the percent of healthy plants three weeks after seeding.

As illustrated in Table 4.2 there was very little difference in the mean percentage of emergence between canola plants with *amp2* integrated into their genome and those without the gene. This indicates that the presence of the synthetic *amp2* gene did not increase the ability of the plants to withstand infection by the fungal pathogen *R. solani*. The difference between the transgenic and non-transgenic plants three weeks after

Table 4.2 Mean percentage of seedling emergence in *B. napus* AW708 in the presence of *R. solani* AG-2-1 [1]

Seed variety	Emergence (%)		
	Range	Mean	+/- s.e.
AW708-1-1	20-53	38	7
AW708-1-3	40-67	52	6
AW708-1-4	13-67	43	11
AW708-1-7	20-40	48	6
AW708-1-9	27-60	30	6
wild type	27-60	42	7

[1] Fifteen seeds were used, and the results were scored after one week of seeding. The values are the average results of four separate experiments. The standard error is expressed as a percentage.

seeding is shown in Table 4.3. The majority of the *amp2* plants were "mildly infected", "severely infected" or "damped off/dead". These results confirm that the presence of *amp*2 does not increase the resistance of canola plants to *R. solani*.

Table 4.3 Disease rating of six transgenic *B. napus* lines following inoculation with *R. solani* AG-2-1

Plant lines	Rating of Infection (%)[1]			
	H	M	S	DO
AW708-1-1	4	35	39	22
AW708-1-3	13	26	35	26
AW708-1-4	19	23	35	23
AW708-1-7	21	17	24	38
AW708-1-9	6	22	28	44
wild type	0	44	28	28

[1]Data obtained from the seedlings that emerged from the four experiments previously described. The numbers represent the percentage of the seedlings which fell into the four different categories. H-healthy; M-mildly infected; S-severely infected; and DO-damped off, dead, wilted

4.3 Discussion

Plants respond to pathogen attack by the activation of a number of defense mechanisms. Some of the major biochemical responses that are observed during pathogen attack are accumulation of antimicrobial phytoalexins, release of oligosaccharides elicitors of plant origin and elaboration of several different classes of pathogenesis-related proteins with antimicrobial properties. One such class consists of small, highly basic and cysteine-rich proteins whose exact mode of action is currently unknown.

Small cysteine-rich proteins which play an important role in host defense have been isolated from vertebrates (Lehrer *et al.*, 1991; Selsted *et al.*, 1993), invertebrates (Cociancich *et al.*, 1993), fungi (Nakaya *et al.*, 1990) and plants (Terras *et al.*, 1994). Until the late 1980's, molecules displaying antimicrobial activity were predominantly measured against bacterial pathogens of pharmaceutical significance and little effort was directed towards studying their efficiency against plant-pathogenic bacteria and fungi (Rao, 1995). Jaynes and his co-workers (1987) and Casteels and his co-workers (1989) were amongst the first to suggest the use of antimicrobial genes, found in insects, to enhance bacterial disease resistance in plants. The advantages of using such genes were that they were small and showed strong antimicrobial activity against a number of different fungi and bacteria. However, it was found that the proteins encoded from certain insect genes needed to be modified in order for the proper expression of the protein in the plant (Boman *et al.*, 1990).

Since fungal, bacterial and viral diseases contribute significantly to lower yields of agriculturally important crops, several research groups have started to focus on the

genetic engineering of disease and insect resistant varieties through the expression of small antimicrobial peptides. Several of the small cysteine-rich plant proteins isolated have been found to be potent inhibitors of the growth of several plant pathogenic fungi and certain Gram-positive bacteria (Terras *et al.*, 1994; DeBolle *et al.*, 1995). Results such as these clearly suggest that the small potent antimicrobial peptides may be useful candidates for the genetic engineering of plants with enhanced resistance against microbial infection. Thus, the main objective of the overall study reported in this thesis was to determine if it is possible to produce tobacco and canola plants that display an increased level of resistance to certain plant pathogens by introducing a synthetic antimicrobial peptide gene, based on a peptide isolated from the seeds of *M. jalapa*, into the genomes of these plants.

In this study the *amp1* and *amp2* genes were created by synthesizing two oligonucleotides, which consisted of 15 overlapping bases, specific for each gene. The corresponding oligonucleotides were annealed to one another and filled-in to create a complete ORF. The nucleotide sequence of each gene was deduced from the protein sequences of AMP1 and AMP2 which first became available at the Third International Society for Plant Molecular Biology conference (Cammue *et al.*, 1991). A comparison of the protein sequences of AMP1 and AMP2 revealed that they are highly basic and consist of 37 and 36 residues, respectively. Both peptides contain six cysteine residues and differ from each other at only four positions. Despite the close similarity between AMP1 and AMP2 at the amino acid sequence level Cammue *et al.* (1992) found that the two proteins differ markedly in their specific antifungal activity. For example, antifungal assays indicated that AMP2 was approximately 10 times more active than AMP1 and

concentrations of approximately 1-5 μg/mL were required for 50% inhibition of growth. Furthermore, when the antifungal activity of AMP2 was compared with the activity of other antifungal proteins it was found that AMP2 was amongst the most potent antifungal proteins known. Due to the findings of Cammue *et al.* (1992) it was decided that AMP1 would not be as productive as AMP2 and that this study would focus, therefore, only on AMP2.

To determine if the *amp2* gene synthesized in this study encoded a protein with antimicrobial properties it was cloned into an expression vector and assayed against the test microorganism *B. megaterium*. A zone of clearing was observed around the disc containing the protein isolated from the *E. coli* strain carrying the *amp2* gene, but clearing was not observed around the discs containing proteins from the control strains. Since the results from these assays indicated that *amp2* encoded a protein that possessed antimicrobial properties the *amp2* ORF was then transferred to the genomes of tobacco and *B. napus* under the control of an enhanced 35S CaMV promoter using the *Agrobacterium*-mediated genetic transformation. PCR analysis of the *amp*2 transgenics revealed the presence of the T-DNA insert confirming the transgenic nature of these plants. The presence of *amp2* was further confirmed in randomly chosen plants by employing the TA cloning system (Invitrogen) and subsequent sequencing. Mendelian segregation of the *nptII* gene, which is closely linked to the *amp2*-encoding sequence, attested to the meiotic stability of the T-DNA's integrated into the transgenic tobacco and canola plants. However, the integration of the *amp2* sequence into the genomes of tobacco and canola plants did not result in any increased resistance of the plants to pathogenic fungi. Crude protein extracts isolated from the transgenic plants did not

possess any antibacterial activity in either the bacterial or fungal assays performed. Plant cells are known to undergo changes during the tissue culture process and any conclusions related to transgene expression should await the stabilization of the transformants through sexual progeny (Potrykus, 1991). For this reason the F_1 and F_2 plants were also assayed for antimicrobial activity. Unfortunately, negative results were obtained for all of the regenerated tobacco and canola assayed except for the progeny of the tobacco line AW708-1. In order to visualize any antibacterial activity in the plate assays carried out very high concentrations (2-4 mg) of desalted protein extracts from this transgenic line had to be used. However, when the AW708-1-1 tobacco plants were grown in the presence of the plant pathogen *R. solani* they displayed similar results as the other transgenic tobacco lines which did not show any antibacterial activity.

Given these negative results, investigations were carried out to determine why the introduction of the synthetic *amp2* into the genomes of tobacco and canola does not appear to enhance their resistance to certain plant pathogens. PCR and sequencing of the amplified fragment confirmed the presence of the gene in the regenerated plants, but antibodies against AMP2 were not available in this study. It was not possible, therefore, to do western blot analysis to confirm that the protein was being synthesized. Unfortunately, the small size of the protein, 3-4 kDa, also made it difficult to confirm with certainty that the protein was present in extracts isolated from the transgenic plants. From the results of SDS-PAGE which was employed to try to visualize the AMP2 protein it appeared that a small protein was being produced in the transgenic plants, but not the untransformed plants. What is known with certainty is that when *amp2* is present in a bacterial expression vector strong antibacterial activity is observed. However,

antimicrobial activity is not observed when the gene is present in the plants. This lack of antimicrobial activity *in vivo* could be due to a number of factors, some of which were further investigated during the course of this study.

The lack of antimicrobial activity of *amp2 in vivo* due to a possible mutation within the gene, which could lead to a shift in the reading frame, has been ruled out by examining the sequences obtained in the TA cloning experiments. The reading frames of each of the sequences obtained from the randomly chosen transgenic plants through PCR were found to be intact. Since no bases had been altered or were missing the possibility that a frameshift rendered an inactive protein was ruled out.

The expression cassettes used to transfer *amp2* to plants consisted of a duplicated CaMV 35S promoter and an untranslated leader sequence from alfalfa mosaic virus RNA4 (AMV leader). Previous research has clearly indicated that the presence of the AMV leader, in combination with a duplicated-enhancer CaMV 35S promoter, leads to improved high-level constitutive foreign gene expression in transgenic plants (Datla *et al.*, 1993). Therefore, the leader sequence and promoter used in this study should have been adequate to express high enough levels of AMP2.

The possibility that DNA methylation may explain why *amp2* was not expressed in the transgenic plants has also been considered as DNA methylation patterns are frequently associated with the modification of gene expression in plant cells. The C residues commonly methylated are CpG and CpNpG. As illustrated in Figure 4.1, the *amp2* nucleotide sequence created in this study contains 9 possible methylation sites. Thus, it is possible that the expression of *amp2* is being silenced due to methylation of all of these sites. The correlation between DNA methylation and the inactivity of T-DNA

genes was also taken into consideration because it has been found that T-DNA which integrates randomly into the genome becomes methylated at certain integration sites (Amasino *et al.*, 1984). Therefore, certain copies of a transgene become hypermethylated while others remain hypomethylated and transcriptionally active depending on their site of integration into the genome. In this case, it is possible, but cannot be confirmed, that *amp2* integrated into regions where hypermethylation occurs. A comparison of the synthetic *amp2* nucleotide sequence and that of the isolated cDNA clone (DeBolle *et al.*, 1995) has revealed that the *M. jalapa* gene contains seven instead of nine possible methylation sites. This then leads to the question of whether or not the presence of the extra possible methylation sites could render an inactive protein in the plants.

The lack of *amp2* activity observed in tobacco and canola plants was not likely caused by a potentially inefficient translation context present in the construct as this possibility was addressed when creating *amp2* and the necessary constructs for genetic transformation. In higher eukaryotes, including plants, the consensus sequence for the most efficient translation is GCC**G**/ACC<u>AUG</u>**G** (Kozak, 1991). A purine at -3 and a G at +4, relative to A (+1) of AUG, are considered to be very important for efficient translation. In order to successfully clone *amp2* into the *Nco* I of the plant cassette pBI524-14 it was necessary to add an additional amino acid immediately following methionine. To follow the rules of the above consensus sequence the amino acid alanine (GTG) was chosen as the second amino acid and the cassette chosen for this study contained a purine at the -3 position. Therefore, the sequence TCCACCATGGTG conforms to the consensus sequence believed to be required for the most efficient translation in eukaryotes.

Improper folding of AMP2 in the heterologous milieu of the canola and tobacco plants could be responsible for the lack of antimicrobial activity observed. Examination of the cysteine and glycine residues of the amino acid sequence of AMP2 reveals the possible existence of a cysteine-stabilized α-helix motif. This motif was originally reported to occur in endothelin (Kobayashi *et al.*, 1991; Tamaoki *et al.*, 1991) and a mammalian peptide with vasoconstricting activity (Yanagisawa *et al.*, 1988). Later, such a motif was discovered to dictate the three-dimensional structure of insect defense A (Bonmatin *et al.*, 1992). This motif is characterized by the occurrence of the sequences CXC, GXC and CXXXC (where X stands for any amino acid). Examination of the AMP2 protein sequence reveals that it consists of one CXXXC sequence and two GXC sequences fitting nicely, therefore, into the proposed motif. Thus, it is possible that the peptide encoded by *amp2* does not form the required structure within the plant which is required for it to be biologically active. During the course of this study the possibility that the presence of cations in the protein extraction buffer and growth media may inhibit the antimicrobial activity of AMP2 was also examined. It has previously been shown that most antimicrobial peptides are sensitive to cations and that this sensitivity is a very important factor in evaluating the possible contribution such peptides may have towards enhancing the defense response of plants (Roberts and Selitrennikoff, 1990; Cammue *et al.*, 1992; Terras *et al.*, 1992). These studies have shown that even very low concentrations of Ca^{2+} and K^{+} have a significant effect on the antimicrobial activity of peptides such as zeamatin, *Rs*-AFP2 as well as *Mj*-AMP1 and *Mj*-AMP2.

In this study it was hoped that the synthetic *amp2* gene would prove to be a suitable candidate for the development of crops with superior ability to resist diseases

caused by fungi and bacteria. The preliminary results obtained from *in vitro* experiments were very promising, however the fungal challenge assays clearly showed that transgenic plants carrying *amp2* did not exhibit any delay in symptoms or any signs of increased resistance. Future work could be undertaken to try to determine the reason why antimicrobial activity disappears once the gene is transferred to plants. Unfortunately, due to lack of activity that *amp2* has displayed *in vivo* it is not likely that it will be of interest to industry especially since there are several antimicrobial genes available for use that have been found to retain their antimicrobial properties upon transfer to plants (Terras *et al.*, 1995; Rao, 1995; Cammue *et al.*, 1995; Greenland, 1996).

4.4 Summary and conclusions

In this portion of the dissertation an antimicrobial gene (*amp2)* was synthesized and found to produce a protein with an antimicrobial activity when expressed in *E. coli* under the control of a *lac* promoter. This gene was introduced into tobacco and canola plants, and the presence of the *amp2* sequence was confirmed by PCR and subsequent cloning of the amplicon. Protein extracts isolated from transgenic lines and their progeny were found not to display antimicrobial activity when assayed against the test microorganism *Bacillus megaterium.* Removal of ions did not result in antimicrobial activity. However, after assaying several different concentrations of proteins it was found that very low levels of antibacterial activity could be observed when using desalted proteins, in the range of 2-4 mg/50μL of plant extract, isolated from AW708-1 homozygous tobacco plants. Although slight antibacterial activity was observed in one transgenic line the survival rate of *amp2*-containing tobacco and canola plants in the presence of *Rhizoctonia solani* was found to be approximately the same as untransformed parental lines. Thus, the presence of *amp2* did not increase their resistance to a fungal pathogen. Since the transgenic plants produced in this study did not show an increase in resistance to plant pathogens it is necessary that additional studies must be undertaken to determine why *amp2* displays antimicrobial activity *in vitro* but not *in vivo.*

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Appendix A

Table A.1 Plasmids used or constructed in this study

Plasmid	Source	Parental Plasmid	Relevant Information
pHS63	G. Selvaraj and R. Hirji (unpublished)	pMON200	Sp^r; DH5α; C58.
pHS723	G. Selvaraj and R. Hirji (unpublished)	pHS981; pRD400	Km^r;DH5α; G3101
pBI524-14	R. Datla (unpublished)	pBI524	Amp^r; DH5α
pSE380	Invitrogen Corporation	pTrc99A	Amp^r; DH5α
pRD400	Datla *et al.*, 1991	pBIN19	Km^r; DH5α; GV3101
pAW3	2.8-kb *Sal* I-*Eco* RI fragment of pSJ12 (Joshi *et al.*, 1988) was cloned into pTZ19R	pSJ12; pTZ19R	Amp^r; DH5α
pAW70	118-bp synthetic AMP2 ORF was cloned into pSE380 as a *Nco* I-*Bgl* II fragment	pSE380	Amp^r; DH5α

Appendix A continued.

pAW307	OL 493 was used to introduce a *Sph* I site by SDM 347 bp downstream of the *Sal* I site	pAW3	Amp^r; DH5α
pAW379	2.4-kb fragment of containing *chiB* gene was cloned into pTZ19R between *Sph* I and *Eco* RI sites	pAW307: pTZ19R	Amp^r; DH5α
pAW439	OL 696 was used to introduce a *Nco* I site by SDM at the start of the *chiB* ORF	pAW379	Amp^r; DH5α
pAW440	OL 697 was used to introduce a *Bgl* II site by SDM downstream of the stop codon of *chiB*	pAW439	Amp^r; DH5α
pAW444	1.5-kb *chiB* ORF was cloned into the expression cassette of pBI524-14 as a *Nco* I-*Bgl* II fragment	pAW440; pB1524-14	Amp^r; DH5α
pAW445	Expression cassette containing the *chiB* ORF was cloned into pRD400 as a *Xba* I-*Xba* I fragment	pAW444; pRD400	Km^r; DH5α; GV3101
pAW446	Expression cassette containing the *chiB* ORF was cloned into pHS723 as a *Xba* I-*Xba* I fragment	pAW444; pHS723	Km^r; DH5α; GV3101

Appendix A continued.

pAW447	Plasmid pAW444 was digested with *Bam* HI to drop out a 0.8-kb portion of the *chiB* gene. The resulting vector was then religated.	pAW444; pTZ19R	Amp^r; DH5α
pAW448	Expression cassette containing the partially deleted *chiB* ORF was cloned into pHS723 as a *Xba* I to *Xba* I fragment	pAW447; pHS723	Km^r; DH5α C58; GV3101
pAW460	Plasmid pAW444 was digested with *Hind* III. The *Hind* III site was then filled in and the vector religated	pAW444; pTZ19R	Amp^r; DH5α C58; GV3101
pAW461	Expression cassette containing the frameshift mutation within the *chiB* ORF was cloned into pHS723 as a *Xba* I to *Xba* I fragment	pAW460; pHS723	Km^r; DH5α C58; GV3101
pAW707	The ORF of AMP2 was cloned into the expression cassette of pBI524-14 as a *Nco* I-*Bgl* II fragment	pAW70; pBI524-14	Amp^r; DH5α
pAW708	Expression cassette containing the AMP2 ORF was cloned into pRD400 as a *Hind* III-*Eco* RI fragment	pAW707; pRD400	Km^r; DH5α; GV3103