Investigation of a rapid screening method to study the effects of the snowdrop lectin (Galanthus nivalis Agglutinin) on plant pathogens

Popovich, Alexandra Helen

Investigation of a Rapid Screening Method to Study the Effects of the Snowdrop Lectin (*Galanthus nivalis* Agglutinin) on Plant Pathogens

Alexandra Helen Popovich, B.Sc, M.Sc

Department of Biological Sciences, University of Durham

Doctor of Philosophy

2002

Supervisors:

Dr S.A. MacFarlane (Scottish Crop Research International)
Dr J.A. Gatehouse (University of Durham)

University of Durham
Alexandra Helen Popovich
PhD

Investigation of a rapid screening method to study the effects of the Snowdrop Lectin (*Galanthus nivalis* agglutinin) on plant pathogens

ABSTRACT

Two Tobravirus expression vectors were evaluated for the use as a rapid screening method for anti-nutritional proteins against plant pathogens. Accumulation of green fluorescent protein (GFP) and snowdrop lectin gene (*Galanthus nivalis* agglutinin, LECGNA2, M55556) in *Nicotiana benthamiana* by *Tobacco rattle virus* expression vectors was characterized. Virally expressed proteins were detected in leaves (3-14 days post-inoculatiion) and roots (6-24 dpi) by UV (GFP), western blotting and tissue printing. 25 –50 ng of GNA was detected in root extracts. Cross protection was induced by TRV-GFP. Foreign genes inserted in place of TRV RNA2 non-structural genes (2b and 2c) were stably maintained over serial passages. But recombination at remaining 'cross-over' sites may occur. 2D iso-electricfocusing detected a 50-kDa GNA molecule in root and leaf extract. GNA did not confer resistance to root-knot nematodes, although gall by root-knot nematodes (mixed *Meloidogyne* spp. and *M. javanica* Crete line 17) were significantly reduced by 22% in roots infected by TRV-GNA (3.83 sqrt galls and 4.5 sqrt galls respectively) compared to virus-free roots treatment (4.94 sqrt galls, sed 0.398; $p<.025$ and 5.273 sqrt galls, sed 0.2403; $p<.003$ respectively). Effects of GNA on *Aulacorthum solani* was delayed to the second nymph generation (N2). Mean N2 weights feeding on TRV-GNA (0.246 mg ±0.0159; $p<.05$) and TRV-fsGNA (0.212 mg ±0.018; $p<.001$) infected plants were significantly smaller by 15.2% and 26.6% respectively, compared to virus-free treatments (0.290 mg ±0.014). Similar trends were detected for total nymph weights. Low toxicity was related to high quality phloem and ingestion of smaller volumes for normal development (i.e. concentration effect). Decrease in gall by the mixed *Meloidogyne* population and an unexpected toxicity to *A. solani* indicated that truncated GNA was a protein with merolectin properties. The viability of this system as a rapid '*in planta*' expression system is discussed.

Key words: *Tobacco rattle virus, Pepper ringspot virus,* transient virus-based expression vectors, *Meloidogyne,* root-knot nematodes, *Aulacorthum solani,* Glasshouse potato aphid, Snowdrop lectin. *Glanthus nivalis* agglutinin, GNA,

ACKNOWLEDGEMENTS

I am grateful to my supervisor Dr Stuart MacFarlane for the opportunity to work on this project and for the training in molecular techniques. My grateful thanks to my supervisor Dr John A Gatehouse and Dr AMG Gatehouse for the supply of anti-GNA antibodies and for the support given during my writing. Thank you to Dr Trefor Woodford for aphid advice and the provision of the aphid clone for my experiments. Many thanks to Dr Christine Hackett and Dr Jim MacNicol for statistical advice regarding my aphid experiments and to Dr Mark Philips regarding my first series of nematode experiments.

I would also like to thank the glasshouse staff for the provision of plants and seedlings for this work. Also for advice and the use of equipment from Mrs Sheena Lammond and Dr A.N.E. Birch. A special thanks to Walter Robertson who directed me towards this project.

My sincere thanks to Dr Graham Seymour for the constructive comments on my final chapter drafts and the structuring of my thesis. A huge thanks to Dr Jacqueline Marshall for her constant support during the latter stages of my writing, for the best advice I could have ever wished for and for the welcome comments she made on my written work. I appreciate your help very much. Thanks also to Dr Sharon 'salsa-queen' Hall, Dr Liz Harrison, Dr Karl Morrison, Rachel Edwards, Paraskevula Mathas and Tania 'chocolate-buddy' Page for being brilliant friends, fantastic company and for making these months of writing so much more pleasant. A special thanks to Maria for her support and friendship during the 'dark' hours.

Many best thanks to my parents who have been extremely supportive in every way throughout the entire experience. A huge thanks to Karen and Dan for their patience, constructive advice and huge amounts of support despite the appearance of David and Sasha. Thank you to my dearest friends Irina and Kevin for their friendship, support and the fun times. You were truly a breath of fresh air. To Joe for her kind patience, support and motivation.

The present work was funded by a Scottish Office Agricultural and Fisheries Department Grant FF818.

TABLE OF CONTENTS

CHAPTER 2
Materials and Methods

List of Tables

List of Figures

1b gene with GFP linked to a subgenomic PEBV promoter (sgP) (B). Cloning strategy of PepRSV/GNAclone 9 (F) from the cloning vector pT7Blue/GNA (E) to the virus expression vector (F) (MacFarlane and Popovich 2000).

3.1 *N. benthamiana* systemically infected with TRV-GFP at 7 dpi, illuminated under UV light. Arrow identifies the study leaf which shows a uniform distribution of GFP across the whole leaf lamina. Photograph also demonstrates the uniform pattern of GFP expression in roots.

3.2 Western blot of time course of GFP expression by TRV-GFP in *N. benthamiana* leaves. M denotes size marker and v-f denotes a virus-free leaf sample. Lanes are labelled in days postinoculation. Panels 1A and 1B are leaf samples harvested between 4 dpi and 10 dpi and panels 2A and 2B are samples harvested between 11 dpi and 23 dpi. Blots 1A and 2A were probed with TRV coat protein specific antibodies. Blots 1B and 2B were probed with GFP-specific antibodies.

3.3 Detection of local GFP fluorescent lesions, under UV light, on *N. benthamiana* leaf infected by third passage TRV-GFP inoculum after 3 days (ii). Fluorescence was not detected on a mock-inoculated leaf (i), or, after challenging a recovered systemically infected leaf with TRV-GFP inoculum (iii).

3.4 Detection of TRV-GFP RNA2 by RT-PCR. Lane 1 and 6, DNA ladder. Fragments of viral RNA2 amplified from plasmid DNA: lane 2, wild-type PpK20 TRV RNA2 and lane 3, TRV-GFPc. Viral RNA2 amplified from total RNA isolated from infected leaves after 4 dpi (lane 3) and 10 dpi (lane 4)

3.5 Western blot to illustrate differences in GFP expression in *N. benthamiana* by TMV-GFP compared to TRV-GFP. Top panel is leaf extracts. Lower panel is extracts of whole root system excluding hypercotyl. Lane 1, positive control (TRV-GFP at 7 dpi). Lanes 2, uninfected tissue extract. Lanes 3 and 4, TMV-GFP inoculated leaves harvested at 4 dpi and 5 dpi. Lanes 5-8, TMV-GFP infected plants showing systemic fluorescence between 6-9 dpi. Lanes 9-10, spacer lanes. Lanes 11-12, tissue extracts from highly fluorescent study leaves systemically infected with TRV-GFP at 4 dpi and 8 dpi respectively. Lanes were loaded with 12 µl tissue:laemli sample buffer. Blots were probed with anti-GFP antibodies.

3.6 Visualization of the pattern of GFP expression in whole *N. benthamiana* plants when illuminated under UV light. Mock-inoculated plant (left) compared with A) TRV-GFP at 6 dpi (right) and B) PVX-GFP at 9 dpi (right). Red-purple colouration is autofluorescence of uninfected tissue.

3.7 Western blots comparing GFP expression in six individual *N. benthamiana* plants infected with TRV-GFP or PVX-GFP. Blot A is extracts of leaves showing highly fluorescent systemic GFP symptoms. Blot B is extracts of the whole root system excluding the hypercotyl. Lanes 1 and 8, uninfected tissue samples. Lanes 2-7, TRV-GFP infected plants at 6 dpi. Lanes 9-14, PVX-GFP infected plants at 9 dpi.

3.8 Summary of systemic GFP fluorescence in leaves and roots recorded during time-course experiments for TRV-GFP, TMV-GFP and PVX-GFP infections in *N. benthamiana*

4.1 Western blot of *N. benthamiana* plants inoculated with TRV-GNA. Lanes were loaded with extracts of leaf or root extract. Panel A and B, shows samples harvested at 10 dpi from transcript inoculated plants. M denotes protein size markers. C, 250 ng purified GNA protein. VF, virus-free tissue extract. Lanes 4-10, plant 3 leaf (L) and root (R); plant 4 L and R; plant 5 R; plant 6 L and R. Panel A was probed with CP-specific antibodies. Panel B was probed with GNA-specific antibodies. Panel C and D, show samples harvested at 7 dpi from first passage inoculated plants. Inoculum was root extract originating from plant 6. Lane 1, 500 ng purified GNA protein, lane 2, virus-free tissue extract, lanes 3-8, plants 1-6. Blot presented in panel C (leaf extracts) and panel D (root extracts) were probed with GNA-specific antibodies. Root extracts were prepared from the complete root system excluding the hypocotyl.

4.3 Western blot of *N. benthamiana* plants inoculated with transcript TRV-GNA and TRV-fsGNA. Lanes were loaded with equal volumes of root extract. Lanes 1-6, TRV-GNA infected plants at 7 dpi. Lanes 7-12, TRV-fsGNA inoculated plants at 9 dpi. Panels A and B were probed with CP-specific antibodies and GNA-specific antibodies respectively.

4.4 Western blots of extracts of *N. benthamiana* plants inoculated with TRV-ΔGFP, a TRV virus expression vector with an empty expression cassette. Panel A shows first passage extracts (transcript inoculated plants). Lanes V, TRV infected extract. VF, virus-free extract. Lanes 1 – 4, TRV-ΔGFP infected plants at 7 dpi. Mx, mix of tissue extract from TRV and TRV-ΔGFP infected plants. Panel B shows inoculum viability of first passage inoculum (tissue extracts from plants 1-4 shown in panel A). A and B represent duplicate infections. VF, virus-free extract. Blots probed with anti-cp antibodies.

4.5 Non-denaturing two dimensional, isoelectric focusing blot of GNA expressed by the TRV vector in systemically infected leaves and roots at 9 dpi. Extracts were separated using ampholytes ranging from pH 3.5 to 10. Column was loaded with leaf (panel A) and root (panel B) samples respectively. Blots were probed with GNA-specific antibodies.

4.5 Time course of synthesis of CP and GNA in TRV-GNA infected *N. benthamiana* plants. Two systemically infected leaves (panel A and B) and the complete root system (panel C and D) were sampled. Lanes were loaded with equal volumes of sap. VF, virus-free extract. Time course samples are shown as 3-24 days postinoculation. Blots shown in panels A and C were probed with CP-specific antibodies. Blots shown in panels B and D were probed with GNA-specific antibodies.

4.6 Virus-free *N. benthamiana* plantsgrown in sieved sand, in a 30 ml pot (A) at 27 days growth and (B) approximately 40 day growth.

4.7 Western blot of time course of GNA expression by the TRV-GNA expression vector in *N. benthamiana* plants (grown in sand in 30 ml pots). Lanes were loaded with equal volumes of root extract. C, 25 ng purified GNA protein. Fs, root extract from a TRV-fsGNA infected plant. Panel A shows infected root samples at 2-11 dpi. Panel B shows the infected root samples at 12-23 dpi. Plants were transferred from a temperate glasshouse to heated glasshouse at 8 dpi. Lanes were loaded with equal volumes of tissue extract. C, 50 ng purified GNA protein. Fs, root extract from a plant inoculated with TRV-fsGNA. Panel C shows the infected root samples at 3-10 dpi. Panel D shows the infected root samples at 11-23 dpi. Blots were probed with GNA-specific antibodies.

4.8 Tissue print of *N. benthamiana* roots infected with TRV-GNA and TRV-fsGNA at 10 dpi. Plants were grown from seedlings in sand, in 30 ml pots. Roots from mock-inoculated plants (C), TRV-fsGNA infected plants (F) and TRV-GNA infected plants. Blots were probed with anti-CP antibodies (panel A) or anti-GNA antibodies (panel B).

4.9 Tissue print of *N. benthamiana* roots. Plant infected with TRV-GNA at 21 dpi and probed with (A) GNA-specific antibodies and (C) CP-specific antibodies. Wild-type TRV infected plant probed with (B) GNA-specific antibodies and (D) CP-specific antibodies.

4.10 Western blot showing GNA expression by TRV vector over four serial passages in *N. benthamiana* plants. Lanes were loaded with equal volumes of root extract. Lane C is 50 ng purified GNA protein. Lane Fs is root extract from a TRV-fsGNA inoculated

plant. Lane 1 is first passage (7 dpi); lane 2 is second passage (14 dpi); lane 3 is third passage (11 dpi) and lane 4 is fourth passage (9 dpi) tissue extract. Panel A and B were probed with GNA-specific and CP-specific antibodies respectively.

4.11 Sequence alignment of the subdomain regions for GNA (shown in blue, is adapted from Hester *et al.*, 1996). Subdomains I, II and III are flat four stranded, antiparallel β-sheets with individual strands labelled β1-12 (shown underlined). Conserved residues contributing the polar surface of the 3 mannose binding-sites are highlighted in bold (Hester *et al.*, 1996). The translated amino acid sequence of GNA that is shared by the truncated protein is shown in black. To illustrate the change in the amino acid residues after the frame-shift the GNA is shown in blue and the changes in the truncated protein shown in red. Uncoded residues after the prematiure termination codon are represented as*.

4.12 Western blots comparing GNA expression in *N. benthamiana* plants infected with transcript PepRSV-GNA. Panel A and B presents extracts of leaves harvested at 7 dpi. Lane 1, 25 ng GNA. Lane 2, uninfected tissue sample. Lanes 3-7, inoculated plants. Panel C and D presents time-course of GNA and CP expression by PepRSV. Lane 1, PepRSV infected sample. Lane 2, uninfected tissue sample. Lanes 3-12, PepRSV infected plants at 3 to 12 dpi. Panel E presents PepRSV-fsGNA transcript inoculated plants harvested at 7 dpi. Lane M, marker. Lane 1-6 and 7-11, leaf root extracts respectively. Panel A and C was probed with GNA-specific antibodies. Panel B, D and E was probed with CP-specific antibodies. Lanes were loaded with equal volumes of root extract.

5.1 Schematic representation of the rapid *in planta* nematode bioassay experiment showing approximate time taken in days.

5.2 Bioassays 1 to 3. Galling induced by *Meloidogyne* spp nematodes on *N. benthamiana* roots recorded after 10 days. Gall counts are expressed as square root of data. Untransformed data are shown as labels.

5.3 Mean number of galls (sqrt transformed) counted on virus-free *N. benthamiana* roots (V-F) compared with roots infected with TRV-GNA or TRV-fsGNA. Panel A) is *Meloidogyne* spp data for bioassays 4 to 6. Panel B) is *M. javanica* Crete line 17 data for bioassays 7 to 10. Untransformed gall data is shown as data labels.

5.4 A) Pertri-dish of *N. benthamiana* roots galls by the root knot nematode (*Meloidogyne* spp.) 14 DAI. Panels B) and C) show variation of gall development.

5.5 Tissue print of TRV-GNA infected roots showing localization of virally expressed GNA in galls induced by *M. javanica* after 10 days. Root gall are labelled as discrete (D), chains (C) or small (S). GNA was detected by GNA-specific antibodies.

6.1 A schematic representation of the rapid *in planta* Tobacco rattle virus expression bioassay protocol adapted from Gatehouse *et al.*, (1996), including the aphid responses monitored.

6.2 Western blots of TRV-GNA infected leaf tissue sampled on day 7 of the aphid bioassay (sample A) for bioassays 1 to 4 (Blots 1 to 4) and day 12 (sample B) for bioassay 4 (Blot 5). Lanes are loaded with equal volumes of tissue extract; lane 1, 20 ng of GNA protein standard, lane 2-11 contains infected plants 1 to 10. Blots were probed with GNA-specific antibodies.

6.3 Trend of antibiosis of individual *A solani* nymphs measured as weights (μg) after feeding on leaves containing GNA (ng). Accumulated level of GNA was estimated by densitometric analyses. Bioassays are shown as 1, 2, 3 and 4

ABBREVIATIONS

α	alpha
A1	first generation adult
A2	second generation adult
ANOVA	analysis of variance
APS	ammonium persulphate
ASA	*Allium sativum* (garlic) agglutinin
Asn	Asparagine
Asp	Aspartic acid
β	Beta
BS	binding site
°C	degree centigrade
C-	carboxyl
CaMV 35 S	*Cauliflower Mosaic Virus 35 S*
cDNA	complementary DNA
Con A	Concanavalin A
CP	coat protein
CPA	Chick pea agglutinin
Cv	cultivar
Δ	deletion
d	day
DAI	days after infestation
DI	defective interfering
d.f.	degrees of freedom
DMF	dimethylformide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-5'-triphosphste
dpi	days postinoculation
ds	double strand
EM	electon microscope
ER	endoplasmic reticulum
fs	frame-shift
g	gram
Gln	Glutamine
GFP	green fluorescent protein
GNA	*Galanthus nivalis* (snowdrop) agglutinin
h	hour
IEF	iso-electric focusing
IPM	integrated pest management

J2	second juvenile stage
J4	fourth juvenile stage
kb	kilobase
kDa	kilodalton
L	long
LC	lethal concentration
LcH	*Lens culinar* (lentil) agglutinnin
µ	micro
M	molar
µl	micro litre
mM	milli molar
Man	D-mannose
MBL	mannose binding lectin
mg	milli gram
m.s.	mean square
N1	first generation nymph
N2	second generation nymph
nm	nano metre
NPA	*Narcissus pseudonarcissus* (daffodil) agglutinin
N-	amino
NS	non significant
OD	optical density
ORF	open reading frame
OSA	*Oriza sativa* (rice) agglutinin
p<	probability
PEBV	*Pea Early Browning Virus*
PepRSV	*Pepper ringspot virus*
PSA	*Pisum sativum* (pea) agglutinin
PVX	*Potato Virus X*
%	percentage
ref	reference
rep	replicate
RIP	ribosome inhibiting proteins
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
s	second
S	short
SDS	sodium dodecyl sulphate
SDS –PAGE	SDS- polyacrylamide gel electrophoresis

s.e.	standard error
sgpr	sub genomic promoter
sqrt	square root
STD	standard deviation
TE	Tris-EDTA
TEMED	Tetramethyl-ethylenediamine
TMV	*Tobacco Mosaic Virus*
TRV	*Tobacco Rattle Virus*
Tyr	Tyrosine
U	unit
UDA	*Urtica dioica* (nettle) agglutinin
UV	Ultra violet
V	volt
v.r.	variance ratio
WGA	Wheat germ agglutinin
w/v	weight to volume
v/v	volume to volume

Chapter 1

Literature Review

1.1 Applications of lectins in crop protection

Internationally, 37 % of crop yield losses are attributed to plant pathogens annually with 13 % assigned to insect damage (Gatehouse *et al.,* 1993) and 5-10 % due to nematode damage (Barker *et al.,* 1985). Endoparasitic nematodes are estimated to cause losses of $100 billion annually with the root-knot nematode attributing to only 5 % of this damage (Sasser and Freckman 1987; Atkinson *et al.,* 1995; Lilley *et al.,* 1999). Application of chemical pesticides is a habitual management strategy. However, pesticides provide only short-term solutions to the control of insect and nematode pests and often result in resurgence months after application and the selection of resistant races. Other disadvantages associated with pesticide application include: 1) broad-spectrum activity to a large variety of soil organisms including natural parasites (Mankau 1980); 2) high cost especially for small-scale farmers; 3) persistence in soil and contamination of ground water (Niebel *et al.,* 1994), particularly in the case of nematicides which has led to withdrawal from the market and 4) misshapen potatoes caused by granular nematicides and increasing the cropping season (Scholte 1990). Alternatives to chemical control agents like biological control (Jatala, 1986), organic amendments (Halbrendt, 1996) and transgenic crops are being investigated. The primary goal being that these practices would complement conventional forms of crop protection and provide choice and flexibility in a sustainable agricultural system while reducing pesticide usage (Sayre 1980; Gatehouse *et al.,* 1992, 1993; Hilder *et al.,* 1995; Sauvion 1996).

The interest in the monocot mannose binding lectins as a potential tool in crop protection was initiated by speculation and then evidence that their genes conferred resistance to insects and nematodes (Hilder *et al.,* 1995). It was hoped that by using advances in protein isolation and gene transfer technologies it would be possible to utilize lectins, or other naturally produced plant proteins, in the protection of agriculturally important plants (Peumans and van Damme 1995).

1.2 Bioassays are used to screen compounds for pesticidal properties

Lectins and other proteins are usually investigated and screened for potential nematicidal or insecticidal properties in biological assays (bioassays). In nematode studies

bioassays are performed for endoparasites (root-knot nematodes or cyst nematodes) to determine relative population levels in yield loss experiments or nematicide evaluation (Barker, 1985). In insect studies the term bioassay may be defined as the 'measurement of any stimulus by the reaction it produces in living organisms' (Devonshire and Rice 1988). The objective of the bioassay is dependent on the purpose of the assay and candidate pathogen. For crop protection purposes, the criteria for the 'ideal' lectin would include several desirable characteristics: i) anti-insect and anti-fungal activity; ii) pesticidal activity at a concentration below the maximum obtainable level; iii) the active concentration would not affect the vigour of the plant and iv) be non toxic to consumers (Peumans and van Damme 1995). However, other characteristics including low toxicity to non-target organisms and wildlife, low mammalian toxicity and issues related to public acceptability like potential allergenic reactions should also rate highly during the assessment of lectins.

1.2.1 Nematode bioassays

For nematode studies the bioassay generally measures two criteria in the nematode life history. Firstly, root gall indices, assessed on a scale of 0-100, which measures the incidence of root-knot nematodes in the soil. The gall index is recommended for the purposes of nematicide evaluation (Barker, 1985). Secondly, the reproductive factor (R_f) is used to assess host resistance against *Meloidogyne* spp. The R_f [final population P_f / initial population P_i] is dependent on the fecundity of the initial inoculum and the success of subsequent generations to infect the host and reach reproductive maturity (Schmitt and Shannon 1992; Brown *et al.,* 1997; Ogallo *et al.,* 1997; Sydenham *et al.,* 1997). Host plant resistance is usually associated with decreased gall formation, inhibited development to female maturity (Dong *et al.*, 1997) and fewer egg masses (Hussey and Boer 1981; Bouton *et al.,* 1989).

Root gall indices can be detected after 10 to 12 days after infestation (DAI) rather than egg counts that are measured after 60 days (depending on environmental conditions). This former parameter was therefore selected to measure the effects of virally expressed proteins on root-knot nematode infestation for this study.

Screening for nematode resistance has in the past been limited to searching through wild germplasm (Sasser *et al.,* 1983), since commercially viable nematode resistant cultivars had proved difficult to produce in breeding programs. For this reason insect resistant cultivars are also re-screened for nematicidal properties (Urwin *et al.,* 1995). Standard laboratory methods used to evaluate cultivars for resistance to plant parasitic nematodes vary from closed canister, pots, petri dish methods (Mugniery and Balandras, 1989c) to small scale field tests comparing plots containing single plants (Phillips *et al.,* 1988). Traditionally the inheritance of resistance in potato to cyst nematodes is tested in petri dishes by inoculating seedlings with 3-5 larvae and comparing frequency of establishment with a control seedling (Mugniery and Balandras, 1989b). But poor root development means that the percentage of resistant seedlings is often overestimated. A more sensitive test is based on stem cuttings taken from potato seedlings that are rooted in growth medium and then transplanted on to water agar before inoculation with larvae (Mugniery and Balandras, 1989a). However, the choice of method used is dependent on the desired level of resistance designated by statutory regulations since each of the laboratory methods mentioned increase in severity and efficiency respectively. The level of resistance of the cultivar is also affected by the choice of non-resistant control cultivar, nematode population or race, test method and the statistical transformation used for data analysis and subsequent ranking of the cultivar (Mugniery and Balandras, 1989c).

Latterly, transgenic tissue generated from Agrobacterium mediated transformation has allowed major advancement in experimentation on obligate parasites by firstly allowing sterile nematode culture. For example, soybean cyst nematode (Savka *et al.,* 1990). Secondly, the generation of hairy roots expressing putative nematicidal proteins, which can be applied in small-scale nematode bioassays (Urwin *et al.,* 1995). For example, tomato transformed with orizacystatin and tested against *Globodera pallida* (Burrows 1996). However, it is speculated that after the screening of pesticidal genes based on hairy root bioassays, transgenic plantlets regenerated directly from the hairy root tissue or a second Agrobacterium transformation, would require re-screening according to the traditional methods described above to rank the level of resistance of the transgenic cultivar.

Root dips, soil drenches (Marban-Mendoza, 1987, 1989; Gupta and Sharma, 1991; Ponti *et al.*, 1996) and direct treatment of the pre-infective juvenile stage (Davis *et al.*, 1989a, b) are also approaches utilized to assess potential nematicides.

1.2.2 Insect bioassays

For insect studies the bioassay should ideally measure several individual responses from which at least one response can be interpreted to qualify and quantify the differences between the plant treatments (Adams and van Emden 1972).

Anti-feedant compounds are usually assessed for application in plant transformation technology in three phases of bioassays. Firstly, artificial diet studies are used to screen a wide range of compounds for effects on insect feeding or development and the production of a dose-response curve. In the case of the phloem-feeding Homoptera specific adaptations may be incorporated into the bioassay design to simulate *in planta* conditions and address the insect's feeding habit. For example, a short-term bioassay feeding *Nilaparvata lugens* (brown plant hopper) nymphs, liquid diet under pressure, through a membrane (Powell *et al.*, 1993). Secondly, *in planta* assays using whole transgenic plants or leaf discs, permit in-depth long-term, physiological and biological studies. Expression of sufficient antifeedant protein in the phloem is a prerequisite to ensure effective control of phloem-feeding Homopteran pests (Gatehouse *et al.*, 1993; Shi *et al.*, 1994, Hilder *et al.* 1995). Therefore, whole plant assays allow the effectiveness of anti-feedant proteins expressed constitutively by the CaMV 35S promoter (Gatehouse *et al.*, 1993) to be compared to phloem specific promoters like the maize sucrose synthase gene *Sh*-1 (Yang and Russel 1990), or the rice sucrose synthase gene (Wang *et al.*, 1992) which result in localized protein expression in the sieve elements and companion cells. Bioassays for insect species like Lepidoptera are less complex because they consume leaf material. Finally, animal toxicity trials are undertaken. However, this subject is beyond the scope of this review.

Plant biotechnological techniques aimed at engineering novel forms of nematode and insect resistance would therefore benefit from a rapid 'pesticidal gene' pre-screening option. One application of a virus-based transient expression system would be to 'bridge'

the gap between the available *in vitro* screening methods and those based on actual transformed plant tissue. The current research investigates the potential of the virus-based transient expression system as an alternative, *in planta*, rapid bioassay method which could accommodate both leaf and root feeding pathogens including phloem feeding insects.

The following reviews reflect the diversity of this project and aims to cover selected topics that are related to each of the research areas. The first review aims to introduce the role of transgenic plants in crop protection and the methods that are most commonly used to screen potential pesticides. The second review aims to introduce the virus expression system (based on *Tobacco rattle virus* or *Pepper ringspot virus*), the method by which the anti-feedant protein was delivered to the feeding pathogen. A general review for the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), the Glasshouse potato aphid (*Aulacorthum solani*) and the root knot nematode (*Meloidogyne* spp) will also be discussed.

1.3 Transgenic plant technology

Discovery of genes that have the potential application in plant biotechnology have been enhanced by the global effort to determine genome structure, and by advances in biosynthesis, regulation and genetic manipulation of plant products (Herbes and Sonnewald 1999; Dixon 1999). In particular, transgenic plant technology based on recombinant DNA techniques has become a useful tool for introducing novel resistance genes into crop species, especially where natural resistance is limited (Gatehouse *et al.*, 1993). The main advantage of this technique is the potential insertion of single gene traits directly into elite varieties that are already created through plant breeding practices (Horsch, 1993). For example, primary gene products (polypeptides) that have pesticidal properties like enzyme inhibitors, toxins neuropeptides and lectins (reviewed in 1.2) (Goldstein and Porestz 1986; de Ponti and Mollema, 1992). Defence related compounds that are bi-products of metabolic pathways (secondary products) are less amenable to this technique than polypeptides as they are generated using relatively conserved enzymatic pathways (Dixon 1999). The challenge after gene discovery, however, is the analysis of

gene function (Herbes and Sonnewald 1999) and the development of methods for *in planta* functional analysis (Peumans and van Damme 1995; Dixon 1999).

1.3.1 Gene expression in plants

In plant molecular biology two approaches are used to express foreign genes in plants. Firstly, stable insertion into the genome (transgenic plants) achieved in a single plant transformation. Here, the transgenic plant usually expresses a modified protein as nutriments or with pesticidal properties for the purpose of crop improvement (Shivprasad *et al.* 1999). Secondly, transient expression using virus-based vectors (see section 1.1.2) or by particle bombardment which requires treatment of each generation of plants separately. Using viral vectors particularly, plants may be engineered to express large quantities of a high value polypeptide (pharmaceuticals, edible polypeptides or active enzymes) at a lower cost than with conventional microbial and yeast production systems (Shivprasad *et al.* 1999). The market or the objective of the recombinant protein influences the transformation method adopted in industry and research.

1.3.2 Transient gene expression using plant viruses

Advances in viral expression strategies have been accomplished through the availability of genome organization and complete nucleotide sequence information (Bustamante and Hull, 1998). However, the choice of potential virus vectors is largely restricted by the genetic engineering techniques available and currently only single stranded DNA geminiviruses, double stranded non-integrating DNA pararetroviruses and plus sense RNA viruses are candidates for gene expression vectors (Scholthof *et al.*, 1996). Four strategies have been identified to express foreign genes in the virus genome these include; gene replacement, gene insertion, epitope presentation and complementation. 'Gene replacement' (see section 1.1.3) has been specifically identified for use with the Tobraviruses, Bromovirus, Furovirus, and Hordeivirus virus groups and the fundamental principle of this strategy involves the removal of non-essential genes and their replacement with foreign genes (Scholthof *et al.*, 1996). This review will concentrate on two

Tobraviruses (*Tobacco rattle virus* and *Pepper ringspot virus*) where possible, as they are relevant to the present study.

1.3.2.1 The Tobraviruses

The Tobraviruses are one of 25 distinct taxonomic groups of single strand, plus-sense RNA plant viruses (Murphy *et al.,* 1995). The *Tobravirus* genus consists of three members: 1) *Tobacco rattle virus* – the type member (TRV) (Robinson, 1993), 2) *Pepper ringspot virus* (PepRSV) previously known as TRV- CAM (Harrison and Woods, 1966, Robinson and Harrison, 1985, Robinson 1996) and 3) *Pea early browning virus* (PEBV) (Robinson and Harrison, 1985). TRV and PEBV are the most studied of the tobraviruses.

1.3.2.2 Geographical distribution and host range

TRV is distributed throughout Europe, the former Soviet Union, Japan, New Zealand and North America (Harrison and Robinson, 1996). TRV has a broad host range infecting more than 400 species including the economically important crops potato, tobacco, spinach, pepper, sugar beet, ornamental bulbs (*Narcissus* spp) (Harrison and Robinson, 1996) and onion (Udhe *et al.,* 1998). TRV infections in potato are also known as corky ringspot disease (Mojtahedi and Santo, 1999). PepRSV is native to Brazil (Harrison and Woods, 1966) and infects tobacco, tomato, *Capsicum* and *Chenopodium* (Smith 1972).

1.3.2.3 Genome organization

The bipartite genome of the tobraviruses is divided between RNA1 and RNA2, which are encapsidated separately into two rod shaped particles. For TRV, the larger (L) particle measures approximately 180 -197 nm and encapsidates RNA1. The smaller (S) particle measures between 46 -114 nm (Harrison and Robinson, 1996) and encapsidates RNA2. Both particles can be detected in tissue extracts using an electron microscope (EM). For PepRSV L and S particles measure 197 nm and 52 nm respectively (Harrison and Woods, 1966).

1.3.2.3.1 RNA1

The genome organization of RNA1 is highly conserved among the Tobraviruses (Hernández *et al.*, 1995; MacFarlane 1999). Sequencing data for TRV isolate PpK20 and TRV isolate SYM (Hamilton *et al.*, 1987) indicates that there are four coding regions. The 5' proximal open reading frame (ORF) codes for a 134 K protein (helicase) and by read-through of a opal stop codon (UGA) a 194 K protein (RNA dependant RNA polymerase). During virus replication these proteins function as the replicase. The third ORF, or 1a gene, encodes the movement protein which is responsible for cell-to-cell movement (Bocarra *et al.*, 1986; Zeigler-Graff *et al.*, 1991; Hamilton and Baulcombe 1999). The role of the fourth ORF (gene 1b) in RNA1 of TRV PpK20 is uncertain but its deletion reduces virus accumulation in whole plants and protoplasts (MacFarlane 1999). In PEBV, the 1b gene (12 K) may be linked to seed transmission (Wang *et al.*, 1997).

1.3.2.3.2 RNA2

The complete sequence of TRV PpK20 RNA2 was determined by Hernandez *et al.*, (1995). The TRV PpK20 RNA2 genome encodes the coat protein (CP) followed by two additional non-structural genes 2b (40 K protein, Visser *et al.*, 1999) and 2c (32.8 K protein) (see Figure 2.2 A). This is not a typical description of a RNA2 molecule and these additional non-structural genes may vary between 1 to 3 depending on virus and isolate. PepRSV CAM RNA2 has a CP and a partial copy of the 1b ORF (see Figure 2.3) (MacFarlane 1999). In contrast to RNA1, the nucleotide sequence of the RNA2 species is extremely varied. Hybridization experiments indicate that typically some sequence homology exists between TRV RNA1 and RNA2 3' and 5' termini (Robinson and Harrison, 1985; Robinson *et al.*, 1987) and the 3' terminal sequence (500 – 1100 nt) of RNA2 molecules is identical to the 3' region of the RNA1 in the same isolate (Angenent *et al.*, 1989a). The molecular biology of the tobraviruses has been recently reviewed by MacFarlane (1999).

1.3.2.4 Virus replication

Replication of positive sense RNA viruses occurs in the cytoplasm of an infected cell, in overlapping stages, and utilizes the host cell translation apparatus. First, the virus particle uncoats which exposes the nucleic acid for translation. In the early phase of replication the viral RNA serves as messenger RNA and produces proteins required for replication and movement. Later, proteins involved in encapsidation (coat protein) are translated. Replication of the genome then occurs in two stages. Firstly, the genomic RNA strand is used as a template for the synthesis of full-length complementary (negative strand) RNA molecules. Secondly, the complementary strand is used as template for the synthesis of genomic RNA and subgenomic RNAs. The virus-encoded RNA dependent RNA polymerase catalyzes both reactions. Encapsidation of the progeny genomic RNA species is the final stage in the infection cycle (Bustamente 1998). Cell-to-cell movement and long distance movement through the plant occurs via plasmodesmata and phloem respectively (Lucas and Gilbertson 1994).

1.3.3 Identification of virus genes for 'gene replacement'

Annotation of viral gene function has been elucidated from studies of nematode transmission studies and defective interfering (DI) RNAs.

1.3.3.1 Transmission of TRV by Trichodorid nematodes

Trichodorid nematodes belonging to the generas *Paratrichodorus* and *Trichodorus* are the natural vectors of TRV and PEBV (Brown *et al.*, 1989). Virus transmission is dependent specifically on nematode species (Ploeg *et al.*, 1991, 1992) and factors located on the RNA2 molecule like coat protein sequence and non-structural proteins (Ploeg *et al.*, 1993 a b).

1.3.3.1.1 Virus coat protein is involved in nematode transmission

TRV particles from transmissible isolates have been found associated with the cuticle lining of the oesophagus (Taylor and Robertson 1970; Brown *et al.*, 1996) or pharyngeal lining of *P. anemones* (Karanastasi *et al.*, 2000). Several features of the virus

particle have been identified that attests to these observations. Firstly, nuclear magnetic resonance data (NMR) shows a mobile or flexible segment, correlating to the C-terminus of the CP, protruding from the virus particle surface in tobraviruses (Mayo *et al.*, 1993; PepRSV, Brierly 1993) and which can be removed by papain digestion, confirming an external location (Legorburu *et al.*, 1996). Secondly, comparison of CP amino acid sequences from the transmissible TRV isolate PpK20 CP is 90 % identical to non-transmissible isolates, except in the C-terminal region (Hernández *et al.*, 1995). In PEBV deletion of the C-terminal region (15 amino acids) abolishes nematode transmission (MacFarlane *et al.*, 1996). However, the virus CP is not the sole determinant of virus transmission as a recombinant virus produced by replacing the CP of non-transmissible PEBV SP5 with CP from transmissible TRV PpK20 does not promote transmission (MacFarlane *et al.*, 1994, 1995).

1.3.3.1.2 Non-structural RNA2 encoded genes are involved in transmission

Mutation-deletion studies indicate that the presence of the 2b ORF in TRV PpK20 was prerequisite for nematode transmission (MacFarlane *et al.*, 1996, Hernández *et al.*, 1997; Vassilakos *et al.*, 2001; PEBV, MacFarlane and Brown, 1995). Mutation of the 23 K ORF (2c) reduced TRV transmissibility (MacFarlane *et al.*, 1996; Schmitt *et al.*, 1998) although, the function of this protein is not fully understood (MacFarlane, 1999). Nematode transmitted tobravirus isolates that are vectored by the same nematode are analogous. Sequence comparison of isolates vectored by *Paratrichodorus pachydermis* (TRV PpK20) and *T. primitivus* (TRV TpO1 and PEBV TpA56) indicated that isolates transmitted by *T. primitivus* exclusively encoded a 9K protein on the RNA2 molecule. But more importantly this study indicated that the CP and the 2b proteins of TRV TpO1 resembled that of PEBV TpA56 rather than TRV PpK20 (MacFarlane *et al.*, 1999). The CP C-terminus amino acid sequence was also almost identical for the isolates transmitted by *T. primitivus* (MacFarlane 1996).

1.3.3.1.3 Defective interfering RNA2s abolish nematode transmission

Accumulation of DI RNA2s is associated with the elimination of nematode transmission. DI RNA2 species have internal deletions in the structural and non-structural genes and have been found to accumulate during serial passage of TRV Ppk20, in competition with the intact viral genome, following mechanical inoculation to tobacco (Hernández *et al.*, 1996). It has been speculated that transmissible-defective laboratory isolates of TRV, that were maintained by mechanical inoculation, might arise due to the DI RNA phenomenon in the absence of selection pressure (i.e. nematode transmission) (Hernandez *et al.,* 1996). Recently, it was shown that nematodes feeding on transgenic plants expressing DI RNA and infected with TRV PpK20 only transmitted the wild-type virus to healthy bait plants. Thus suggesting that a strategy existed for the tobraviruses which under field conditions, would eliminate DI RNAs from the population (Visser *et al.*, 1999).

1.3.4 The Tobravirus-based expression strategy

The transient gene expression using the virus vector system meets several criteria supporting its application as a rapid screening method in this present study. Firstly, the amenability of the tobraviruses for potential engineering is exemplified by evidence of genetic recombination. Stable pseudorecombinants between TRV and PEBV occur naturally, in mixed infections, by re-assortment of the genome parts viruses (intergenic recombination) and thus shows compatibility between the genomes (Robinson *et al.*, 1987). Secondly, virus replication is possible under laboratory conditions despite internal deletions in structural and non-structural genes (Hernández *et al.*, 1996) and, in the case of TRV PLB, deleted sequences between the RNA2 5' and 3' termini (Angenent 1989b). For TRV isolate PpK20 (the isolate used in this research) deletion-mutation studies specifically indicated that both the ORF of 40 kDa and 32.8 kDa proteins encoded by RNA2 were dispensable for replication (Hernández *et al.*, (1995).

Thirdly, one unique feature of the tobraviruses that is useful for the construction of a virus vector is the ability of RNA1 to produce a systemic, NM-type infection (non-multiplying), independently of RNA2. Nucleotide sequence analysis indicates that this is

possible because all genes coding for proteins necessary for virus replication and movement are located on RNA1. However, the absence of RNA2 (and CP) means that RNA1 is not encapsidated in this infection and therefore NM- type infections cannot be identified by electron microscopy (Harrison and Woods, 1966, Harrison and Robinson 1982; MacFarlane 1999). Consequently, the RNA2 molecule is a potential target for gene replacement as nonstructural proteins are not essential for the establishment of a viral infection.

Lastly, a major advantage of the virus-based expression vector is that after construction of the virus vector, mature plants can be inoculated thus minimizing the effects of phytotoxic proteins that may interfere with plant growth and development. Transient expression of high levels of foreign gene products is attained when the foreign mRNA is amplified during virus replication. Accumulation of the foreign protein is rapid and detectable within 2 weeks post inoculation allowing the evaluation of expressed protein (Shivprasad *et al.* 1999) or the initiation of bioassays. In contrast, transgenic plants regenerated from an Agrobacterium transformation would not be available for several months, which suggests that the former virus-based system would be more rapid and less labour intensive system.

1.3.4.1 The Tobacco rattle virus expression vector

The TRV expression vector used in this present research was based on the replacement strategy and engineered by Stuart MacFarlane, SCRI (MacFarlane and Popovich 2000). The vector was initially designed to express the green fluorescent protein (GFP), in place of the deleted non-structural proteins (2b and 2c) (see Figure 2.2). A previous study by Mueller *et al.*, (1997) found that the CP promoters from TRV and PEBV virus were interchangeable, and transcription of the GFP gene in the TRV/K20/GFP clone was linked to a PEBV CP promoter. Insertion of an additional virus promoter into the viral vector avoided sequence duplication and therefore prevented homology driven instability. The resulting TRV-based virus vector expressed GFP in infected cells thus allowed direct vizualization of the spatial and temporal patterns of virus movement *in planta* (MacFarlane and Popovich 2000).

1.4 Introduction to plant lectins

Plant lectins have been reviewed previously by Etzler, 1985; Peumans and Van Damme, 1995a, b; Pusztai and Bardocz, 1995; Van Damme *et al.,* 1998. The following discussion is therefore a general overview of the structure and function of plant lectins concentrating particularly on the snowdrop lectin, *Galanthus nivalis* agglutinin (GNA), a representative of the monocot mannose-binding lectin group, as it is the subject of the following research.

1.4.1 Plant lectins

Plant lectins are a heterogenous group of proteins which are found throughout the plant kingdom (Goldstein and Poretz, 1986) and in several different plant tissues including roots, storage organs (bulbs, rhizomes and corms), bark, flower, fruit and seeds (Peumans and van Damme, 1995a, b). Related plant species generally contain similar or closely related lectins and consequently lectins have been grouped into seven evolutionarily related families according to their specific carbohydrate-binding properties. These are as follows: 1) legume; 2) chitin binding; 3) Type 2 RIPs; 4) monocot mannose-binding (see section 1.4.4); 5) phloem; 6) amaranthim and 7) jacalin (Peumans and van Damme, 1995a, b)

The term 'lectin' has been previously used synonymously with 'agglutinin' or 'phytohaemagglutinin'. However, recently the term was re-defined by Peumans and van Damme (1995a) as, "all plant proteins that possess at least one noncatalytic domain that binds reversibly to a specific mono- or oligosaccharride". Four types of lectins were distinguished according to the structure of the primary translation product (Peumans and van Damme, 1995):

- *Merolectins* are small, simple monovalent polypeptide proteins with a single carbohydrate-binding site that do not agglutinate cells or precipitate glycoconjugates;

- *Hololectins* are constructed exclusively from carbohydrate-binding domains and contain at least two identical or homologous domains that bind to the same or similar sugars. This

group characteristically agglutinates cells or precipitates glycoconjugates and contains the majority of the plant lectins;

- *Chimerolectins* are fusion proteins containing a carbohydrate-binding domain(s) tandemly arrayed with a second domain with a different biological activity. The agglutination activity of chimerolectins is dependent on the number of sugar-binding sites and they are only classed as lectins (hololectin-like) if multiple carbohydrate binding sites are present (Peumans and van Damme, 1995; van Damme *et al.*, 1998);

- *Superlectins*, exists as a special class of chimerolectins which occurs as a fusion protein containing two tandemly arrayed carbohydrate-binding domains. Each domain is structurally different and recognizes unrelated carbohydrates (van Damme *et al.*, 1998).

1.4.2 The monocot mannose-binding lectins

The monocot mannose-binding lectins are a superfamily of evolutionarily related proteins isolated from the monocotyledonous Amaryllidaceae, Alliaceae, Araceae, Orchidaceae and Lilliaceae families (Van Damme, 1992; Barre *et al.*, 1996; Van Damme and Peumans, 1995, 1996). This group of lectins are encoded by large families of closely related genes which share over 80 % sequence homology and therefore share common features like biosynthesis, structure and functional properties which are apparent within the taxonomic groups (Van Damme *et al.*, 1992; Van Damme and Peumans, 1990b, 1996). The phylogenic relationship of this lectin superfamily has been deduced from amino acid sequence comparison, and demonstrates that the highest homology exists between the Amaryllidaceae and Alliaceae, followed by the Orchidaceae which are more related to the former families than the Araceae and Lilliaceae families (Barre *et al.*, 1996). The snowdrop lectin (GNA) (Amaryllidaceae) is similar to lectins isolated from the Alliaceae (tribe Allieae) and Orchidaceae (merolectins) families (Van Damme *et al.*, 1987; 1991c, Hester *et al.*, 1995).

1.4.2.1 The snowdrop lectin

The mature GNA lectin is a homo-tetramer assembled from subunits with a molecular mass of 12.5 kDa if determined by SDS-PAGE, or, 11.72 kDa if based on the amino acid sequence composition (Van Damme *et al.,* 1987, 1991c,a). The GNA molecule is described in greater detail in section 1.4.2.2.1. However, lectins like GNA are actually present in tissues as complex mixtures of isolectins (Van Damme *et al.,* 1991a) which are related multiple forms of a lectin, that have different isoelectric points, but which are encoded by a family of genes (Van Damme and Peumans, 1990b). The different isoelectric points are apparent at the molecular level where a change in amino acid residue(s) alters the charge along the polypeptide. The occurrence of multiple lectin isoforms are therefore not detectable by SDS-PAGE (Van Damme *et al.,* 1991a) but are detectable by ion-exchange chromatography and iso-electric focusing (GNA, *Narcissus pseudonarcissus* (daffodil) agglutinin (NPA), Van Damme *et al.,* 1988; amaryllis, Van Damme and Peumans, 1990b, 1991a, 1992).

Studies of snowdrop and daffodil indicate that the isolectin composition of each tissue is unique and developmentally regulated. For example, snowdrop bulbs contain a few very acidic isolectins whereas the ovary tissue contains isoforms with a higher isoelectric point (Van Damme and Peumans, 1990b; Van Damme *et al.,* 1991b). Five isolectins (LECGNA 1, 2, 3, 5 and 8) have been isolated from developing snowdrop ovary cDNA libraries. Oligonucleotide screening demonstrated that LECGNA3, and 5 were the most abundant, each representing 30 % of the total lectin clones, whereas LECGNA2 was only 5 %. Phylogenetic analysis based on amino acid sequence homology illustrated the relatedness of LECGNA3 to 5, and LECGNA2 to 1 and 8 (Barre *et al.,* 1996). However, according to nucleotide sequence alignment, LECGNA2 is highly homologous to LECGNA3 and 5 (Van Damme *et al.,* 1991b). The cDNA for LECGNA2 was used in the present research (see Chapter 2).

Analysis of cDNA libraries from Amaryllidaceae species shows that the coding regions of the isolectin genes are highly homologous. For example, the signal peptide (Van Damme and Peumans, 1990b; GNA, NPA and *Hippeastrum* hybrid (Amaranth) agglutinin (HHA), Van Damme *et al.,* 1992). Van Damme *et al.,* (1992) found that

snowdrop and daffodil cDNA clones were similar at the nucleotide (70 - 86 %) and amino acid (66 - 88 %) level, with sequence conservation at certain regions. The C-terminal showed higher variability with respect to length and sequence. However, Northern blot analysis indicated that the mRNAs were the same size (Van Damme *et al.*, 1992).

1.4.2.2 The biosynthesis of lectins

The processing steps that occur between the primary translation product and the mature polypeptide are complex and differ depending on the individual lectin. Generally, lectins are synthesized as pre(pro)prolectins on the ER and the signal peptides of 'all' lectins is cleaved off co-translationally upon transport across the ER (Van Damme *et al.*, 1991a, b; Van Damme and Peumans, 1996). Glycosylation may occur in the ER, but usually proteolytic cleavage (at N- or C-terminal or internal peptides), deglycosylation or glycan chain modification occurs after leaving the ER. Carbohydrate analysis reveals that Amaryllidaceae and Alliaceae lectins are not glycosylated (Van Damme *et al.*, 1988; 1991c).

Van Damme and Peumans, (1996) describe five types of post-translational processing that differ in complexity, but which result in molecularly and structurally similar forms of the mature lectins. Firstly, removal of a C-terminal peptide, which describes the processing of GNA and NPA (nonglycosylated < 50 amino acid residues), hevein and nettle (glycosylated and >50 amino acid residues). Secondly, removal of an internal peptide (for example, Ricin). Thirdly, removal of both the first and second processes (for example, *Allium sativum* agglutinin and *Arum maculatum* agglutinin). Fourthly, removal of an N-terminal peptide, 1 and 2 (for example, jacalin, *Artocarpus integrefolia*). Lastly, the processing of Concanavalin A (Con A) which is unique. The polypeptides are then transported to the target organelle (vacuole-derived organelle) via the Golgi system (Van Damme *et al.*, 1991a, b; Van Damme and Peumans, 1996).

1.4.3 The physiological role of plant lectins

In planta, lectins have been associated with several physiological roles during plant development.

1.4.3.1 Lectins as storage proteins

Lectins like GNA are similar to classic storage proteins in that they are structurally homogeneous secretory proteins, synthesized on the rough endoplasmic reticulum (ER) and stored in vacuoles of storage tissue with other storage proteins (Van Damme *et al.*, 1991a, b; Van Damme and Peumans, 1996). Secondly, GNA (and NPA) and storage proteins are biochemically similar as they contain high levels of asparagine/aspartic acid, glycine, serine, leucine but are low in cysteine residues (Van Damme *et al.*, 1991a).

Evidence from the snowdrop life cycle demonstrates that accumulation and utilization of GNA in the snowdrop bulb is developmentally regulated like storage proteins. The bulb is an underground storage organ containing compounds including carbohydrates and proteins. At the resting stage GNA constitutes 15 % total protein but at the time of flowering this has decreased to 1 %. The GNA decreases during plant development as the stored compounds are converted into simple carbohydrates and amino acids and utilized for rapid growth. However, at the end of the growing season the nitrogen compounds are salvaged and re-accumulate in the new bulb scales (Van Driessche 1988, Van Damme and Peumans 1990). In perennial bulbs the old scales die off and form scales around the new bulb (Peumans and Van Damme, 1995b). This pattern of lectin accumulation is also characteristic of legume seeds, where lectins are synthesised in the cotyledons during seed development and degrade on the onset of germination (Van Driessche 1988; Van Damme and Peumans, 1995) and is also apparent in vegetative storage tissue like the rhizomes of ground elder (Peumans *et al.*, 1985), and the bark of elderberry or black locust (Nsimba-Lubaki *et al.*, 1986).

1.4.3.2 A role in ovary development

GNA has been isolated from leaf, stem and flower tissue (Van Damme and Peumans 1990a; Van Damme *et al.*, 1992). High quantities of mRNA have been detected in ovary and leaf tissue by northern blotting although only low levels have been detected in the flower tissue (Van Damme *et al.*, 1991b). It is possible to speculate that low levels of GNA in the flower parts may have evolved to protect insect pollinators. Previous studies

on wheat (Triplett and Quartrano, 1982) and rice embryos (Peumans and Stinissen, 1983; Stinissen *et al.,* 1984) indicate that lectin synthesis was under the control of the plant hormone abscisic acid and that the presence of lectin was associated with the suppression of embryo development. It is therefore possible that GNA may have a similar role in the snowdrop ovary.

1.4.3.3 A role in plant defence

1.4.3.3.1 In bulbs lectins are storage proteins with passive defence properties

One explanation for the ubiquitous presence of GNA in snowdrop (Van Damme and Peumans 1990a, Van Damme *et al.,* 1992) is a role in plant defence. Investment of defence substances, like anti-microbial compounds, are common in long-lived tissue and are associated with plant tissues that are important or vulnerable (Etzler, 1985; Peumans and Van Damme, 1995). It has previously been established that GNA is a major storage protein in bulbs (see section 1.4.3.1). Peumans and Van Damme, (1995a) proposed that in storage organs, lectins are passive defence proteins as well as a nitrogen source. Therefore, both physical defences (bulb scale) complemented by chemical defences (lectins) prevent fungal penetration, or pathogen attack, and promote survive of dormant bulbs in the degrading soil environment. This suggests that the roles of storage protein and defence compounds are not conflicting and may have co-evolved in snowdrop and other similar species. Anti-fungal properties have been associated with nettle lectin (Broekaert *et al.,* 1989) and hevein (Van Parijs *et al.,* 1991).

1.4.3.3.2 *In planta* lectins provide a constitutive defence system

GNA has been isolated from developing leaves, stem and flower tissue (see section 1.4.3.2). These observations may imply that GNA is multifunctional, for example, like in ovary tissue (see section 1.4.3.2). Or, that the snowdrop plant is vulnerable throughout its life cycle and therefore dependent on lectins as a constitutive defence system. Herbivores, microorganisms or insects are attracted to nutrient rich tissues (Edwards, 1989). The snowdrops' growing season is early spring (between mid January and mid February) which may increase a plants' apparencey in the environment (Feeny, 1976). Multiple

isolectins may provide a broad-spectrum of activity against phytophagous pests (Van Damme *et al.*, 1992).

Insecticidal effects have been demonstrated for a range of lectins isolated from wheat germ (WGA) (Murdock *et al* 1990), rice and stinging nettle (Huesing *et al.*, 1991), pokeweed (Czapla and Lang, 1990) and garlic (Hilder *et al.*, 1995). Ricin, a ribosome inhibiting protein (RIP) is extremely toxic to eukaryotes. However, Gatehouse *et al.*, (1990) found that ricin was ineffective against two lepidoptera species (*Spodoptera litoralis* and *Heliothis virescens*), this was an early indication that lectin toxicity might not be universal. Research was concentrated on the snowdrop lectin because early feeding trials indicated a low toxicity towards mammals (Pusztai, *et al.*, 1990) which suggested that this lectin was an ideal candidate for transgenic crop technology. Insecticidal properties of GNA have been demonstrated in artificial feeding bioassays for the rice brown planthopper (*Nilaparvata lugens*), pea aphids (*Acyrthosiphon pisum*), sugarcane white-grubs (*Antitrogus parvalus*), Glasshouse potato aphid and Peach Potato aphids (*Myzus persicae*) (Powell *et al.*, 1995; Rahbé *et al.*, 1995; Allsopp and McGhie, 1996; Down *et al.*, 1996; Sauvion *et al.*, 1996). Also from transgenic studies on tobacco and potato (Hilder *et al.*, 1995; Gatehouse *et al.*, 1996). The anti-feedant effects of GNA to Homoptera are reviewed further in section 1.6.2.

In conclusion, lectins may be a group of multifunctional proteins that provide an economic, constitutive defence system in growth conditions that are not conducive to biosynthesis (Vining, 1990). Lectins as recognition molecules and their binding specificity to oligosaccharides, foreign glycans or complex animal glycoconjugates (Peumans and van Damme, 1995; van Damme *et al.*, 1998), in relation to plant defence, is discussed below.

1.4.4 Lectins as recognition molecules

The roles or function of lectins *in planta* have not been clearly defined, although their fundamental physiological role is likely to be attributed to the unique carbohydrate-binding properties and specificities of an individual lectin (Peumans and van Damme, 1995b). This idea forms the basis for the proposal that lectins, because of their wide range

of carbohydrate specificities, are multifunctional proteins with the potential to function as 'recognition molecules' (Peumans and van Damme, 1995a, b). Evidence supporting this proposal is dispersed between several fields of research but can be considered at three main levels.

1.4.4.1 The sugar code

Firstly, monosaccharides and oligosaccharides have the capacity for information storage that is estimated as being more than seven-fold greater than that possible for peptides and therefore they provide a code system for molecular events involved in intra- and intercellular recognition processes (Gabius 2000 abstract, 2001). In cells the sugar code (covalently linked monosaccharides or glycans) is a means of information which, when deciphered by receptor molecules like endogenous lectins, guides the intra- and inter-cellular movement of glycoproteins, regulates cell proliferation and migration and mediates cell adhesion. The coding capacity of oligosaccharides has only recently been fully appreciated (Solis *et al.*, 2001).

1.4.4.2 Lectin-monosaccharide specificity is dependent on binding-site architecture

Secondly, the specificity and precision of the match between a macromolecule and its receptor molecule is determined by the formation of non-covalent bonds between the interacting surfaces and the match of atoms on these surfaces (Alberts *et al.*, 1989). For lectins, binding affinity is associated with both their own surface architecture and also the great diversity of carbohydrate structures, which result from the sequence of monosaccharide, linkage position, ring size, branch points, substitutions and anomeric configuration (α or β) of the glycosidic units (Sharon and Lis, 1989, Laine 1997 abstract).

Glycan binding studies have confirmed that the plant lectin recognition process is complex in that some lectins have a specific monosaccharide receptor molecule, while others have a more complex specificity with a higher affinity to oligosaccharides containing monosaccharide chains (van Damme *et al.*, 1998). For example, although the monocot mannose-binding lectins are highly specific to mannose, GNA preferentially

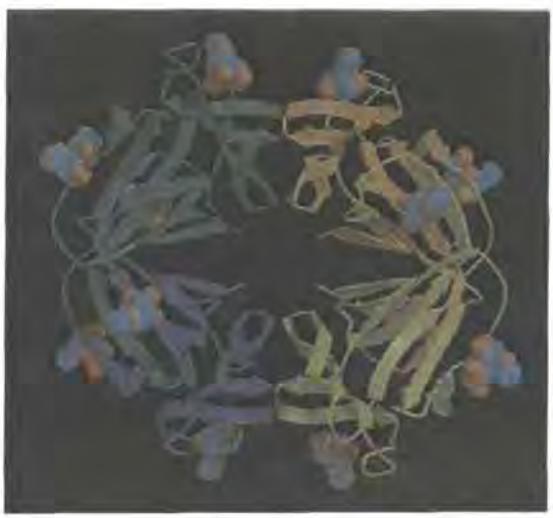
links to terminal mannose α-1,3-linked mannosyl (Oda and Minami 1986), the daffodil lectin (NPA) recognizes both internal and terminal α-1,6-linked mannosyl, the amarylis lectin recognizes both α-1,3 and-α-1,6-linked mannosyl (Kaku *et al.*, 1990) and the tulip lectin (Liliaceae) is inhibited by both L-fucose and D-mannose but preferentially links to α-1,6-linked mannosyl-only oligosaccharides (Shibuya *et al.*, 1988). However, the mannose-containing oligosaccharide thyroglobin is a more potent inhibitor of GNA than NPA than compared to a monosaccharide (Van Damme *et al.*, 1988), although binding affinities are weak compared with some legume lectins (Kaku and Goldstein, 1992). Hapten inhibition assays also indicate that carbohydrate concentration is an important factor during glycan binding. For example, greater levels of mannose were required to inhibit Alliacea and *Clivia* lectins compared to GNA, NPA or HHA (Van Damme *et al.*, 1991c).

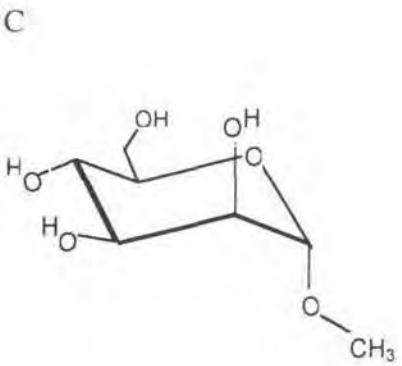
The differences in discrimination by lectins for single monosaccharides and larger oligosaccharides is related to an extended binding site where affinity of the primary determinant is significantly increased depending on the formation of additional hydrogen bonds with the second sugar unit (Weiss and Drickamer, 1996). Therefore although the amino acid residues that are responsible for the binding of mannose are phylogenetically conserved in the monocot mannose-binding lectins (Barre *et al.*, 1996), the fine differences in sugar specificities in this lectin superfamily are related to an extended architecture of the mannose binding site and non-conserved regions (Van Damme *et al.*, 1992).

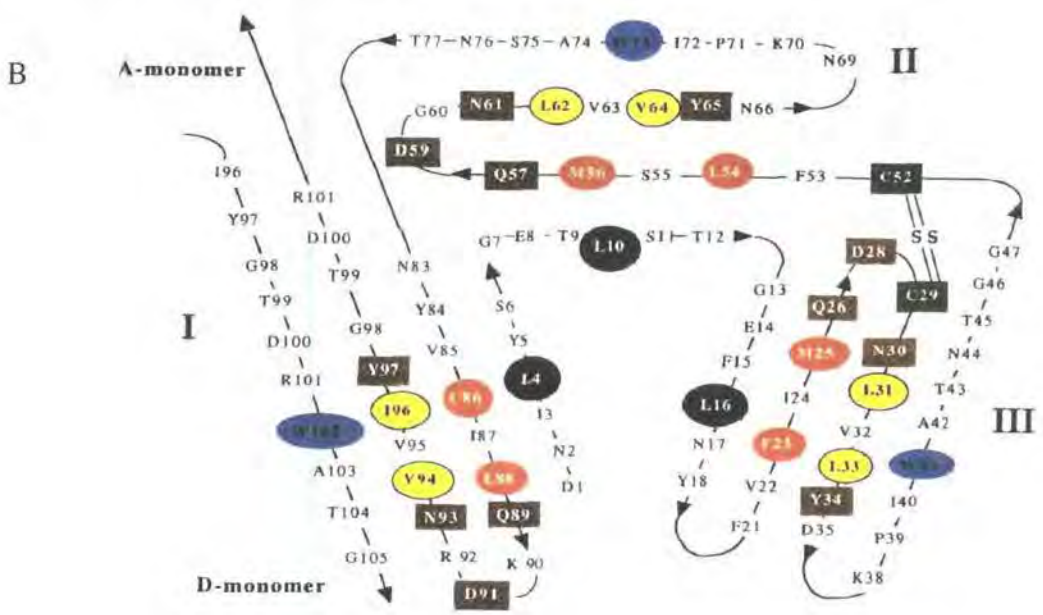
1.4.4.2.1 Description of the GNA molecule

Information based on X-ray crystallography, electron density mapping (Hester *et al.*, 1995, Hester and Wright, 1996) and molecular modelling (Barre *et al.*, 1996), indicates that the GNA polypeptide (monomer) "has a unique conformation consisting of three sequential β-sheet subdomains (I, II and III) that are inter-related with three-fold symmetry" (Hester *et al.*, 1995). Each of the three subdomains is four-stranded, anti-parallel β-sheets. Subdomain I forms the interfacial region between subunits A-D and B-C, although it is unusual as it is a composite of both the N-terminal and C-terminal regions

Figure 1.1 A) GNA tetramer substituted by Methyl-α-Mannose at all 12 binding sites. The crystallography independent monomers are distinguished by colour. B) Schematic illustration of the GNA fold. The three antiparallel four-stranded B-sheets, which form a 12-stranded B barrel, are labelled I, II and III. Sheet I is a hybrid composed of three strands of subunit A and the fourth strand borrowed from the C-terminal end (97-109) of a 2-fold-related subunit. C) Stereo view of Methyl-α-Mannose (taken from Hester *et al.*, 1995, Hester and Wright, 1996).







of the polypeptide (see Figure 1.1A) (Hester *et al.*, 1995). The latter subunit pairs are interlinked by hydrogen bond contacts, but stable dimers are formed through C-terminus exchange where the strands are inserted into the β-sheet 12 of subdomain I. The C-terminal arm from the opposing subunit encircles the saccharide complexed at the mannose binding site (BS I), which is formed in the interface of subunits A and D (B and C), resulting in a high affinity binding site (Hester *et al.*, 1995). Interactions between the narrow interface of subunits A-B and C-D are related to two well-ordered water molecules which co-ordinate H-bonding between two residues (N17 and G19) on opposing subunits. These latter residues are conserved among the Amaryllidaceae lectins. The GNA tetramer can therefore be described as 'two loosely associated dimers' (Hester *et al.*, 1996). A ribbon drawing of the GNA tetramer is presented in Figure 1.1 B and shows that GNA is composed of four independent, flat shaped subunits, termed A, B, C and D and associate with a central (hydrophillic) channel (drawing copied from Hester *et al.*, 1995).

1.4.4.2.2 The saccharide binding site

The carbohydrate recognition properties of GNA were based on its molecular structure complexed with a variety of saccharides. Electron density mapping indicated that each subunit could accommodate three mannose residues (twelve per tetramer). All binding sites were conserved as four residues (Asp, Asn, Gln and Tyr) at the β-turn of the two highly conserved inner strands in the 3-fold internal repeat structure (Hester *et al.*, 1995, Hester and Wright, 1996). These residues were found to be highly conserved in all Amaryllidaceae, Orchidaceae lectins and the majority of Alliaceae, but not in Lilliaceae and Araceae lectins (Barre *et al.*, 1996). Results from the saccharide crystal complexes revealed that BS I (β-sheet I) was the most specific for reducing mannose or terminal non-reducing (methyl-α-mannose, see Figure 1.1 C). The degree of occupancy at BS II and III is low (30 – 50 %) and is dependent on auxillary contacts between neighbouring molecules, which is a function of the lattice environment at each individual site (Hester *et al.*, 1995). BS III has an extended binding region complementary to α-1, 3 linked mannose oligosaccharides, and the reducing end of the bound di-mannoside is situated in

a conserved pocket surrounding the binding site (Hester and Wright, 1996). Weak methyl-α-mannose binding was observed at all three binding sites but the most highly occupied site was located at subdomain III in subunit D. Strong binding of di-mannoside was only observed at sites on subdomains II and III (Hester *et al.*, 1995). The analysis indicated that GNA had unique functional properties, which suggested that GNA was a powerful multi-site lectin that "utilized multiple binding modes to complex oligo mannan receptors" and which had the ability to adapt to diverse environments (Hester *et al.*, 1995, Hester and Wright, 1996).

1.4.4.3 The role of lectins in recognition of 'self' and 'non-self'

Thirdly, glycoproteins, glycolipids and polysaccharides, consisting of sugars including mannose, fucose (6-deoxygalactose) and glucosamine, are present in plasma membranes (Grimes and Breidenbach, 1987), the tonoplast (Dozolme *et al.*, 1995), the endoplasmic reticulum and dictyosomes (Harrison and Chesterton, 1980, Cook, 1986, Salisbury and Ross *et al.*, 1992). The scope for inter-cellular communication for glycosylated membranes is therefore wide. However, despite the widespread presence of glycosylation, the variation and discriminative ability of this code is exemplified in agglutination tests which are evidence of cell-surface recognition in animal cells and thus can be used to examine the differences in cell surface glycoconjugates. For example, GNA agglutinates rabbit but not human erythrocytes (Van Damme *et al.*, 1987) but will agglutinate human leucocytes (Fenton, 1999). In mammals, pathogenic microorganisms are recognized from their surface carbohydrates and then by the macrophage mannose receptor which subsequently mediates endocytosis (Napper *et al.*, 2001). In humans, it has recently been shown that "deficiency of the innate, humoral immune component mannose-binding lectin (MBL) predisposes individuals to a variety of infections" and it was suggested that resistance to MBL is associated with more commonly pathogenic bacteria (Townsend *et al.*, 2001). It has been speculated that similar cell recognition processes are involved in the detection of invading pathogens in plants (Gibson *et al.*, 1982; Dixon and Lamb, 1990; Hester *et al.*, 1995) and also root surface recognition by non-pathogenic rhizobial bacteria (Bohlool and Schmidt, 1974). In conclusion, there is evidence to support

the concept that the sugar code is a universal language for recognition molecules which highlights the potential application of lectins like GNA as a tool for mimicking or blocking of natural ligands.

1.5 Endoparasitic root pathogens

1.5.1 Nematodes pests in agriculture

The target root pest for this part of the study is the root-knot nematodes (*Meloidogyne* spp.) which are a serious agricultural pest in the tropics and sub-tropical regions; for example, *M. incognita* (Kofoid and White, 1919) and *M. javanica* (Treub, 1885; Jepson 1987; Niebel *et al.*, 1994). These parthenogenic nematodes affect a variety of crops including field, vegetable, ornamental and weeds with the most severe damage being observed in light and sandy soils (*M. incognita* and *M. javanica,* Ibrahim and Rezk, 1986). However, some plant parasitic interactions are highly host specific and management practices require identification of *Meloidogyne* spp and race (Sasser *et al.*, 1983). Root gall symptoms are the characteristic damage caused by this species of nematode. Although the level of galling observed is not necessarily related to the initial population density at planting, as good host plant quality and favourable environmental conditions are conducive to 1 to 3 overlapping generations and high fecundity (approximately 2000 eggs in 65 days per female at 26°C) resulting in large damaging populations over a single cropping season (Trudgill *et al.*, 1992; *M. javanica,* Tzortzakakis and Trudgill, 1996).

1.5.1.1 Biology and life history of *Meloidogyne* spp nematodes

Meloidogyne nematodes are obligate plant endoparasites that spend a significant part of the life cycle within roots (Niebel *et al.*, 1994). Firstly, a sedentary, mature, globular-shaped female deposits single-celled eggs into a gelatinous matrix (the egg mass) which protrudes from the root. The juvenile nematode develops within the egg, moults, and then exits the egg as the second juvenile stage (J2) using its stylet mouthparts. The infective J2 nematodes are then attracted to the growing root by a gradient of plant exudates. Root invasion generally occurs in the region of root elongation located distally/proximally to the root tip or, at a site of a new lateral root (Clark and Moyer,

1988). The J2 then migrates non-destructively and inter-cellularly toward the root apex/tip, where the absence of differentiated endodermis allows migration into the differentiating vascular cylinder (*M. incognita*, Sijmons *et al.*, 1991; Wyss and Grundle, 1992; Neibel *et al.*, 1994). Nematode secretions then induce cell re-differentiation at the chosen feeding position, which results in cell hypertrophy due to repeated mitosis without cytokinesis. This process eventually forms a complex of multi-nucleate giant cells called a coenocyte which form the feeding site (Jones and Payne, 1978; Subbotin, 1993; Niebel *et al.*, 1994; Williamson, 1999). The giant cells function as nutrient sinks, or transfer cells, and divert solutes through several feeding tubes from the xylem, phloem and surrounding cells (vascular system) (Jones and Northcote, 1972; Rebois *et al.*, 1975; Jones, 1981; Subbotin, 1993, Niebel *et al.*, 1994).

The gall continues to swell and the developing juvenile nematodes increase in volume until the J4 stage which then moults into the adult stage (Taylor and Sasser, 1978; Niebel *et al.*, 1994). High levels of extensin, an abundant structural wall protein, have been detected in the gall cortex and pericycle cells (Carpita and Gibeaut 1993). This has been thought to be involved in increasing plant cell rigidity and therefore resistance to the dividing plant cells and growing nematodes (Neibel *et al.*, 1994).

Females remain sedentary and continue to enlarge until they form a globular shape at maturity after which egg deposition occurs. The mean number of eggs per egg mass was 700 and 1000 for *M. incognita* and *M. javanica* respectively (Barker *et al.*, 1985). Parthenogenetic reproduction is a universal feature of *Meloidogyne* spp. nematodes. For example, exclusive mitotic parthenogenesis has been observed for *M. javanica, M. incognita* and *M. arenaria* (Triantaphyllou, 1960), while facultative amphimixis and meiotic parthenogenesis has been observed for *M. graminis, M, graminicola, M. naasi, M. ottersoni* and frequently for *M. hapla* (Triantaphyllou, 1979). In amphimictic species the male nematodes metamorphose into a long vermiform state within the fourth stage cuticle and migrate out of the root to fertilize the female nematode (Sijmons *et al.*, 1994). In the latter, typically sex is determined by nutrition. For example, the proportion of developing males is higher compared to females when the quality of the host plant as a food source is suboptimal (McClure and Viglierchio, 1966; Davide and Triantaphyllou,

1967; Bird 1960), when the root is crowded (Triantaphyllou, 1960) or when juveniles developed in lateral roots (Mugniery 1977).

1.5.2 Mechanisms of lectin toxicity

1.5.2.1 Lectins bind to carbohydrates on the surface coat, amphidia and amphidial secretions

The surface coat (glycocalyx) is a secreted coating on the nematodes' cuticle. Wheat germ agglutinin labelled with fluorescein isothiocynate has been used to label the second stage infective dauer larvae producing a micro fingerprint when visualized under blue or UV light (Bird 1988). Similar banding patterns have been observed for amphid secretions suggesting that these observations are species and race specific (McClure and Stynes, 1988; Davis *et al.,* 1988). Evidence to support this has been demonstrated from several lectin-binding studies. Blocking the glycocalyx surface with competitive sugars followed by lectin treatment revealed variation in carbohydrate binding domains at the head, amphids or amphidial secretions and pore openings of nine different species of plant parasitic nematodes (Robertson *et al.,* 1989).

Parallel studies for *Meloidogyne* species indicate that several lectins including Con A and WGA, *Lotus tetragonolobus* agglutinin and *Limulus polyphemus* agglutinin bind to the amphidial openings and secretions of the *M. incognita* and *M. javanica* J2 stage (Davis *et al.,* 1988). Accessible N-acetyl-D-glucosamine residues have also been identified in the amphid and buccal secretions of *M. javanica* J2 (Bird *et al.*, 1989) and compared to *Anguina tritici* more lectins bound to the amphids and amphid secretions of *M. incognita* J2 (Speigel and McClure, 1991). Binding of Con A to the surface of all stages of *M. javanica* demonstrates a predominance of mannose/glucose residues (Ibrahim, 1991). Protein patterns from homogenates and body wall extracts of *M. incognita, M. arenaria* and *M. javanica* J2 are also species specific. Further, detergent-soluble-proteins isolated from the body walls of *M. incognita* race 1 and race 3 J2 were found to selectively bind Con A after analysis by western blotting thus allowing typing of pathogenicity (Davies and Kapland, 1992). Although, lectin labelling experiments were not stringent enough for the characterization of Mi resistance breaking *Meloidogyne* lines, but

strains of *Pasteuria penetrans* could differentially recognize some of the resistant populations (Fargette *et al.*, 1994).

The glycocalyx is a mutable surface. The age-related lability and transitory qualities of the glycocalyx have been demonstrated in studies where *P. penetrans* adhesion to the surface of pre-infective *M. javanica* juvenile stage (J2) ceases after moulting to the male or female form (Stirling *et al.*, 1986), where Con A (Concanavalin A) binding is variable during J2 development but binds entirely to the female cuticle (Ibrahim, 1991), and in changes in adhesiveness to human red blood cells (Speigel *et al.*, 1997). This is a significant observation as a transgenic plant expressing lectins may be stage specific or only be viable for a short period in the nematode life history.

The glycocalyx surface plays a primary role in pathogen attachment. The carbohydrate recognition domains on the cuticle of *M. incognita* J2 have been shown to interact with the N-acetylglucosamine moieties on the spore surface of *P. penetrans* allowing attachment (Davies and Danks, 1993). Exposure of spores to WGA, Con A or glycocalyx extract (Speigel *et al.*, 1996) or treatment of *M. Javanica* with WGA has been shown to inhibit or reduce *P. penetrans* attachment to the J2 surface (Bird *et al.*, 1989).

1.5.2.2 Nematicidal properties of lectins

The present knowledge of the nematicidal activity of lectins to the root-knot nematode is been based on several studies. Application of low doses of Con A solution over consecutive weeks significantly reduced infestation of tomato by *M. incognita* (Marban-Mendoza, *et al.*, 1987), but application of ground *Canavalia ensiformis* beans were not nematicidal (Marban-Mendoza, *et al.*, 1989). Extracts of garlic bulb have shown ovicidal properties at 0.05 to 10 % (reduction in egg hatch by 88.64 to 98.88 % respectively) and larvicidal properties at 1 to 5 % (20.67 and 88 % receptively) for *M. incognita* after 24 hours. However, it was found that extracts from leaves were less toxic (61.33 %) after the same period (Gupta and Sharma, 1991).

Galling was reduced when tomato plants were co-cultivated with *C. ensiformis* plants. It was proposed that nematode control was related to root leachate containing Con A entering the soil (Marban-Mendoza, *et al.*, 1989). Significant reductions in root gall

were also observed when tomato was co-cultivated with the tropical legumes *Pueraria phaseoloides* and *Arachis pintoi*. Anti-nematicidal properties were related to soluble lectin homologues present in the soil detected by ELISA (Marban-Mendoza, *et al.*, 1992).

Two studies by Davis *et al.*, (1989ab) indicated that incubating second-stage juveniles of *M. incognita* in lectin (including soybean agglutinin, Con A) or sugar solutions prior to inoculation on to soybean did not prevent galling. Although Con A treatments did moderately reduce fecundity for *M. javanica* and *M. incognita* race 3 (Davis *et al.*, 1989).

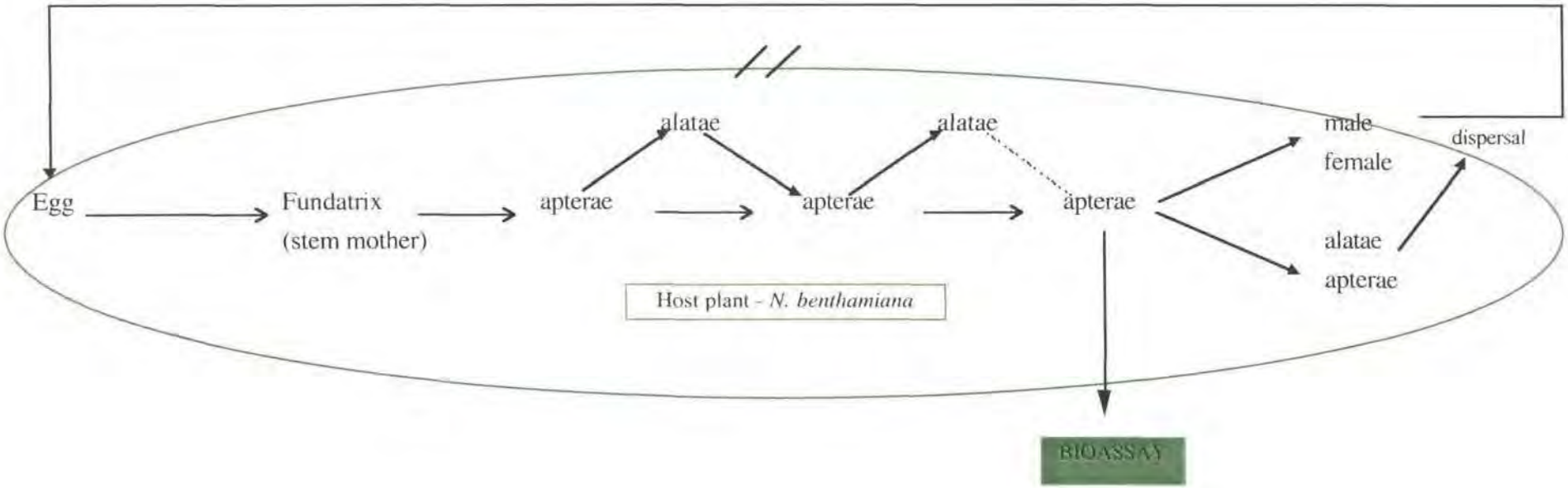
1.6 Leaf feeding plant pathogens

1.6.1 Aphids as pests in agriculture

The target pest for this current study is the Glasshouse potato aphid, *Aulacorthum solani* (Kaltenbach) [Homoptera: Aphidae], which is one of the most polyphagous, non-host-alternating aphid species (Dixon 1998). *A. solani* is a major problem in crops of potato and *Capsicum* spp, but is also a minor pest of buckwheat (*Fagopyrum esclentum*), carnation (*Dianthus caryophyllum*), tobacco (*Nicotiana tabacum*), tomato (*Lycopersicum esculentum*) and causes minor damage to the sprouts of stored potato tubers (Hill, 1987).

Members of the order Homoptera are sap sucking pests that feed by probing plant tissue for phloem elements with their fine stylet mouthparts (Auclair 1963; Evert *et al.*, 1968). Large populations, in particular, cause plant stress by nutrient diversion and cell damage. Honeydew (excreta) deposition may also encourage growth of secondary pathogens like black sooty mildew (Guldemond *et al.*, 1998). However, more significantly, aphids constitute the largest group of plant virus vectors. *A. solani* is known to transmit more than 30 viruses including several persistent viruses: *Soybean dwarf virus*, *Bean leaf roll virus*, *Legume yellows virus* and *Beet western yellows virus* (Thottaphilly *et al.*, 1990). But other species like *M. persicae*, for example, is known to transmit approximately 120 viruses (Thottaphilly *et al.*, 1990). In the past a wide range of systemic insecticides have been applied to control Homopteran pests. However, their effectiveness is limited due to the rapid evolution of resistance-breaking bioforms (Devonshire, 1989). This outcome is partially due to the removal of natural predators from the environment

Figure 1.2 A schematic representation of the life cycle of *Aulacorthum solani*, a monoecious, holocyclic aphid species (adapted from Minks and Harrewijn, 1987). The *A. solani* clone was continuously reared on *Nicotiana benthamiana* plants. Sexual alatae were observed in the culture due to culture conditions of 19 °C, 16h light at suboptimum light levels.



after pesticide application (mentioned in Gatehouse *et al.*, 1993; Powell *et al.*, 1993). In the case of planthoppers (Delphacidae) and leafhoppers (Cicadellidae), problems with control are due to the possession of detoxification enzymes like non-specific esterases (Chang and Whalon 1987; *N. lugens* and *N. cinciteps*; Powell *et al.*, 1993). Integrated pest management (IPM) methods are therefore favoured to optimize insect control and it is thought that protection offered by transgenic plants would be a compatible component in an IPM method as it would permit greater flexibility during crop protection in a sustainable agricultural system (Hilder *et al.*, 1995).

1.6.1.1 Biology and life cycle of the Glasshouse potato aphid *A. solani*

The Scottish clone of *A. solani* used in this research was distinctive and easily identified by a conspicuous bright green region located on the abdomen at the base of each siphunculus. The life cycle of this aphid is 'holocyclic' and 'monoecious' in that a sexually reproducing generation exists on the secondary host, but that there is an absence of host alternation (Minks and Harrewijn, 1987). A clone is originally initiated by the fundatrix (stem mother) which produces the first parthenogenic generation. The parthenogenic phase allows the aphids to multiply to pest proportions. Subsequent generations of aphids are alternately apterae (asexual) and alatae (sexual) morphs, and these differ morphologically as wingless or winged morphs respectively (polymorphism). Usually in holocyclic life cycles the alate morphs are produced in the autumn (instead of the apterae) in response to sap, photoperiod or crowding (Dixon 1985; Minks and Harrewijn, 1987). Alatae are responsible for colonising new hosts, for example, the primary host plant where the over-wintering egg form is produced (Kindlemann and Dixon, 1989). It has been previously speculated that wingless males present in monoecious aphid species like *A. solani,* have evolved because of the absence of migration (Dixon 1985; Minks and Harrewijn, 1987). The life cycle of *A. solani* is illustrated in Figure 1.2.

1.6.2 Mechanisms of lectin toxicity

The mechanisms of lectin toxicity in insects are not well understood and may be related to multimechanistic effects (Powell *et al.*, 1995, 1998) which suggest that any classification may be arbitrary. The possible routes of lectin toxicity to homopterans, and possibly other insects, are summarized in Figure 1.3.

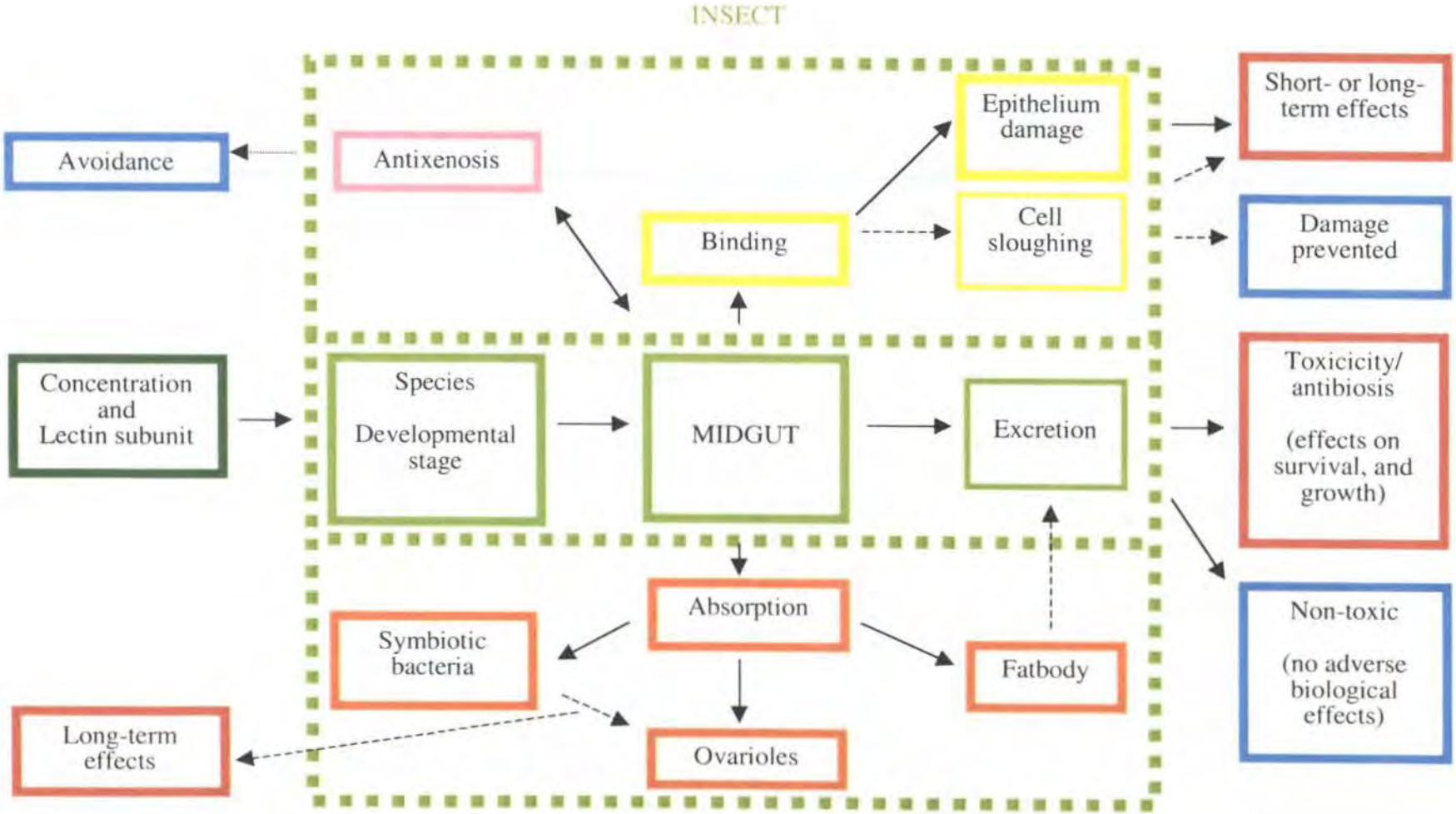
1.6.2.1 Mannose-binding lectins are most toxic to hompteran insects

In section 1.4.4 it was established that the fundamental physiological role of a lectin would be attributed to its unique carbohydrate binding properties and the association with the surface properties of glycosylated membranes with which it contacts. No generalization can be made regarding the sugar specificity of a lectin and insecticidal properties (Rahbé *et al.*, 1995) as lectin toxicity may be affected by the fine specificity's of the binding-sites enabling the lectin to differentiate between highly complex structures (Kaku and Goldstein, 1992; Harper, *et al.*, 1995; Powell *et al.*, 1995b). Insect bioassays have shown that the most effective lectins against Homopteran pests are the mannose-binding lectins, particularly GNA. Toxicity in this lectin group, for some homopterans, has been related to the number of subunits per molecule. For example, for *N. lugens* the LC_{50} values for GNA (tetramer), the daffodil (*Narcissus pseudonarcissus*) lectin NPA (trimer) and garlic (*Allium sativum*) lectin ASA (dimer) were 4 μM (0.02 % w/v), 11 μM (0.04 % w/v) and >40 μM (0.1 % w/v) respectively (Powell *et al.*, 1995 b). A similar trend in lectin toxicity has also been observed for some aphid species like *A. pisum* (Pea (*Pisum sativum*) agglutinin PSA, lentil (*Lens culinar*) agglutinnin LcH, Con A and Amaryllidaceae lectins, Rahbé *et al.*, 1995) and *M. persicae* (GNA, NPA, ASA, Sauvion *et al.*, 1996). Many lectins including OSA and UDA which bind to N-actelyglucosamine residues of chitin were non-toxic (0.1%, *N. lugens*, Powell *et al.*, 1995b).

1.6.3 Anti-feedant effects are detected after lectin ingestion by homopteran insects

The antimetabolic effects of several lectins have been measured in insect bioassays as a negative or positive effect on the pest biology, for example, growth, fecundity and survival. However, data from the following studies suggest that the homopteran-lectin

Figure 1.3 Multimechanistic routes of lectin toxicity to Homotera. Dashed lines represent possible pathways.



relationship is highly complex. Factors that may contribute to the toxicity of GNA to insects are discussed including insect species, ecology, pre- and post-ingestion.

1.6.3.1 Lectin toxicity is related to insect species

Data from lectin dose response curves suggest that aphid sensitivity may be dependent on feeding habit and host-range characteristics (Rahbé *et al.*, 1995). Polyphagous aphids like *M. persicae* (Sauvion *et al.*, 1996) that feed on a wide range of food plants are the most resistant to lectins including ConA (Rahbé *et al.*, 1995). Whereas, oligophagous aphids like *Acyrthosiphon pisum* and *Macrosiphon albifrons* (Rahbé *et al.*, 1995) or *N. lugens* (Powell *et al.*, 1998) that are restricted to the Fabaceae or rice (latter) are the most sensitive. Recent evidence from host plant choice tests, also suggests that diet acceptability is species specific since *N. lugens* nymphs avoided transgenic rice constitutively expressing GNA, whereas *Nephotettix virescens* nymphs (the Green Leaf Hopper) found the same plants attractive (Foissac *et al.*, 2000). This behavioural difference was attributed partly to increased probing in *N. lugens*, which in plants constitutively expressing GNA, enabled the insect to discriminate against these plants and control plants during tissue probing.

1.6.3.2 Developmental stage of an insect may affect susceptibility to a lectin

Growth stage of the insect may influence susceptibility to lectins. In the case of *A. solani*, the effect of GNA on fecundity was greatest when the nymphs were exposed to GNA throughout their development to maturity (Down *et al.*, 1996). Artificial bioassays indicated that survival of the first and third instar *N. lugens* nymphs were significantly reduced after exposure to GNA, WGA and enzyme soybean lipoxygenase (LPO), whereas only the third instar of *N. virescens* was similarly affected (Powell *et al.*, 1993).

1.6.3.3 Homopteran pests may tolerate the presence of lectins in phloem

There was speculation that GNA may bind to glycoproteins present at chemoreceptor sites, thus, interfering with host plant and diet recognition (Powell *et al.*, 1995a, 1998; Foissac *et al.*, 2000). However, no evidence has been found to support the

role of GNA as a universal feeding deterrent for homopterans. Studies of *N. lugens* have found that exposure to GNA reduced the number and volume of honeydew droplets by 96 %. This anti-feedant effect was less pronounced after the ingestion of WGA and LPO, but with all three cases the insects recovered after 24 hours and then tolerated the diet (Powell *et al.*, 1995a). A transient anti-feedant effect was also observed during short-term exposure to GNA to *N. lugens* (Powell *et al.*, 1998). An initial 'aversion effect' was also observed for *M. persicae* when feeding on transgenic potato expressing GNA (Gatehouse *et al.*, 1996).

Honeydew immunoassays have shown that *A. pisum* (Rahbé *et al.*, 1995), *M. persiace* (Shi *et al.*, 1994) and *N. lugens* (Powell *et al.*, 1993, 1998) excrete GNA. However, recovery of feeding behaviour by homopterans is unlikely to be related to detoxification as GNA is highly resistant to proteolysis (Powell *et al.*, 1998). Little evidence exists for the proteolytic degradation of lectins except for partial proteolysis of PSA (Gatehouse *et al.*, 1994) indicating that these insects have a limited capacity to process polypeptides. Protease inhibitors are effective against lepidopteran larvae but not homopteran pests, as the latter do not rely on proteolysis for nutrition (Gatehouse *et al.*, 1993; Powell *et al.*, 1993). In fact, no trypsin-like, or chymotrypsin-like enzymes have been detected in any aphid tissue (digestive tract), although amino peptidase activity exists in the mid-gut and crop regions of an aphid digestive tract (Rahbé *et al.*, 1995). The absence of proteolysis therefore supports the evidence that detoxification mechanisms are absent in aphids (Rahbé and Febvay 1993, Rahbé *et al.*, 1995). Some lectins, including WGA, chickpea (CPA) and Con A, were not excreted or digested by *A. pisum* (i.e. no low molecular weight lectin-specific protein fragments were detected). For these lectins it was assumed that an interaction had occurred within the digestive tract (Rahbé *et al.*, 1995). Differences in digestive physiology may account for some of the observed differences in lectin toxicity.

1.6.3.4 Antimetabolic effects of lectins are not dependent on gut interactions

The midgut epithelium is the primary site of nutrient absorption exposure. The predominant cells are columnar absorptive cells that are lined by a brush border and goblet

cells, the site of ion transport, which are distributed between the columnar cells (Chapman 1978; Loeb and Hakim 1996). Previously authors have speculated that GNA and other mannose-binding lectins bind to specific gut epithelial glycoproteins or digestive enzymes (Gatehouse *et al.*, 1984, 1989; Powell *et al.*, 1993; Eisemann *et al.*, 1994). However, there is evidence to show that strong binding of a lectin to the gut membrane is not an accurate indicator of toxicity. For example, ConA and GNA were toxic to *A. pisum* but only GNA was detected in honeydew. This difference was related to the binding specificity's of each lectin and to the small number of glycopolypeptides present in the gut that could interact with GNA (Sauvion *et al.*, 1995). GNA also bound to membrane proteins of *Ostrinia nubilalis* Hüber (European corn borer) and *Diabrotica virgifera virgifera* LeConte (Western corn rootworm) but insecticidal effects were only recorded for the latter (Harper *et al.*, 1995). Differences here were again related to the structure or biochemical composition of the peritrophic membrane, or to the number of receptors present depending on the developmental stage of the insect (Harper *et al.*, 1995).

Immunological studies of *N. lugens* midgut revealed strong binding of GNA to the luminal surface of the midgut epithelial cells. This binding resulted in the disruption of microvilli and abnormalities of the epithelium, which probably interfered with nutrient absorption, nutrient balance and therefore delayed nymph development. However, GNA was also found in the fat bodies, the ovarioles and throughout the haemolymph suggesting that the effect of GNA could be more widespread (Powell *et al.*, 1998). A link between avoidance and the observation that *N. lugens* nymph guts contain more binding receptors for GNA than *N. virescens* nymphs, which are attracted by GNA (Foissac *et al.*, 2000) has not been established. It was also recently established that ferritin was the most abundant GNA-binding polypeptide in the midgut of *N. lugens* specimens (Du *et al.*, 2000). This protein is also associated with the gut, haemolymph and the fat body. These latter observations suggest that the mode of action of lectins might be resolved by further study of these areas.

1.6.3.5 Lectin toxicity may be quantitative

Further experimental evidence supporting the multimechanistic mode of action was presented by Rahbé *et al.*, (1995). In this study, aphid growth inhibition curves were stepped, which might indicate the presence of two phases of toxicity (particularly for *M. persicae*), or the presence of different classes of physiological targets that display different thresholds of inactivation. For example, it has been previously shown that although high concentrations of GNA were more deleterious to the growth and fecundity of *M. persicae* (Sauvion *et al.*, 1996), *A. pisum* (Rahbé *et al.*, 1995) and *A. solani*, (Down *et al.*, 1996). Sublethal doses (10 μg ml^{-1}) of GNA, while adversely affecting fecundity, significantly increased the growth rates of *M. persicae* aphids which would have implications for aphid control by transgenic crops (Sauvion *et al.*, 1996). Similarly, 1.5 % WGA significantly reduced the survival of female adult *Empoasca fabae* whereas 1.0 % WGA did not (Habiba *et al.*, 1993). In studies of the sugarcane whitegrub *Antitrogus parvulus* the response to lectin ingestion was cumulative with a lag of 14-day between weight loss and mortality (Allsopp and McGhee, 1996).

Quantitative analysis of *N. lugens* honeydew indicated that while levels of ingested GNA (2.1 μM) caused significant mortality it was also excreted, whereas the most significant absorption of GNA occurred when ingested at low levels (Powell *et al.*, 1998). Toxic effects have also been observed when lectin exposure altered gut fauna. For example, cell lysis and gut damage was associated with bacterial proliferation at the midgut epithelium microvilli surface in GNA fed *N. lugens*. It was not known whether the bacteria were normally present or were opportunistic pathogens (Powell *et al.*, 1998). Lectin-induced blockage of pores in the peritrophic matrix of *Lucilia cuprina* larvae (Blowfly) also resulted in reduced food intake and growth (Eisemman *et al.*, 1994)

1.6.3.6 Toxicity may be related to the absence of midgut cell sloughing

In other insects the midgut is also a entry site of toxins including *Bacillus thuringiensis* (Bt) (Feredici 1993) and viruses (Hoover *et al.*, 2000). *Trichoplusia ni* and *Heliothis virescens* larvae (Noctuidae) can prevent establishment of polyhedrosis disease by clearing primary infections by sloughing off infected midgut cells (Engelhard and

Volkman, 1995; Washburn *et al.*, 1998). SEM studies have also shown that mid-gut tissue of *Manduca sexta* exposed to sublethal levels of Bt can slough-off allowing replacement by stem cell multiplication (Spies and Spence, 1985), and similarly for *Corcyra cephalonia* larvae (Chang *et al.*, 1985). An absence of a cell sloughing mechanism in *N. lugens* may therefore increase the site of entry for some lectins, prolong the period of exposure and increase associated cell damage. Epithelial cell distension, enlargement and shedding were also observed in *A. pisum* after the ingesting of Con A in artificial diet (Rahbé *et al.*, 1995). One possible mechanism of lectin toxicity may therefore be related to the insect being physiologically unable to recognize the lectin as a cue that would stimulate midgut cell sloughing, since the insect is not evolutionarily adapted to feed on that lectin.

1.6.3.7 Toxicity may be related to affects on symbiotic bacteria

Some aphid species rely on symbiotic micro-organisms (*Buchnera* spp) to provide essential amino acids like tryptophan (*Acyrthosiphon pisum*; Douglas and Prosser 1992) and methionine (*M. persicae*; Douglas, 1990) during their diet of phloem. Yeast-like symbionts may also provide sterol precursors for *N. lugens*, although they are not obligatory for normal development (Fredenhagen *et al.*, 1986; reported in Powell *et al.*, 1998). The vital requirement of symbionts in aphids was shown when chlortetracycline treated *A. pisum* nymphs (clone Ox-2) could not survive on tryptophan-free diets (Douglas and Prosser 1992). Symbiotic bacteria are found in bacteriocytes located in the aphid haemocoel (reported in Wilkinson and Douglas 1998) and in similar tissue in leafhoppers. In both cases symbionts are transmitted transovarially to progeny (Chen *et al.*, 1981). GNA has been detected in the haemocoel fat bodies and ovarioles of *N. lugens* which may suggest that, after absorption through the midgut, GNA may bind to the yeast's cell walls or accumulate in the fat body for detoxification (Powell *et al.*, 1998). Damage to symbiotic bacteria in some homopterans may therefore also contribute to deleterious affects associated with lectin ingestion. Speculation that the number of bacteriocytes present in the adult aphid is determined during larval development or maternally at birth (Wilkinson and Douglas 1998) may also provide an alternative route for long-term effects of lectins.

In conclusion, there is evidence from several authors to support the concept of multiple mechanistic contributing to lectin toxicity and anti-feedant effects.

1.7 AIM

The primary aim of this thesis was to produce a rapid method of screening pesticidal genes against plant pathogens. Experiments were designed to investigate the viability of plant viruses as a transient gene expression system that was compatible with both nematodes and insect pests (aphids). This method would potentially be a second '*in planta*' bioassay system where lectins or polypeptides could be assessed prior to production of transgenic plants.

Chapter 2

Materials and Methods

2.1 Oligonucleotide primers

166 5' CGGCCAAACGCCGAATTCAAACAGTC 3'

Complementary sense primer that binds to the 3' non-coding region of TRV PpK20 RNA2 (position 3639 – 3656)

250 5' GAGCATAATTATACTGATTT 3'

Positive sense primer, CP subgenomic promoter derived from PEBV/A56 RNA2 (position 393 – 412)

254 5' GGTGGAATCAGTTTCGTGG 3'

Positive sense primer, TRV PpK20 RNA2, CP gene (position 625 – 643)

300 5' CGAGAATGTCAATCTCGTAGG 3'

Complementary sense primer, binds to 3' non-coding region of TRV PpK20 RNA2 (position 3534 - 3555)

305 5' GGGCGTAATAACGCTTACG 3'

Complementary sense primer, universal first strand cDNA primer for the genomic RNAs of tobraviruses PEBV and TRV, binds to the 3' termini sequence.

319 5'ATTTGTTTTTATGTTCAGGCGG 3'

Positive sense primer, 2b gene of TRV PpK20 RNA2 (position 1522 – 1543)

2.2 Gene cloning techniques

2.2.1 Extraction and Purification of Plasmid DNA

The alkaline lysis method (Sambrooke *et al.*, 1989) was used for small scale plasmid DNA purification.

2.2.2.1 Bacterial culture preparation

Single colonies of bacteria (*Escherichia coli*) containing the plasmid of interest were inoculated into Luria-Burtani medium (1 L of LB contains; 10 g bacto-tryptone, 5 g

bacto-yeast extract, 10 g sodium chloride at pH 7; Sambrooke *et al.*, 1989) and 50 μg/ml ampicillin, unless specified, and grown in a rotary incubator overnight at 37 °C.

2.2.2.2 Bacterial glycerol stock

Aliquots (500 μl) of glycerol were dispensed into screwtop tubes and autoclaved. An equal amount of overnight bacterial culture was then added and vortexed to mix. Tubes were frozen in LN_2 and then stored at -70 °C.

2.2.3.1 The purification of plasmid DNA by alkaline lysis

A 1.5 ml volume of overnight culture (2.2.2) was centrifuged at 14 000 *g* (Eppendorf ™ centrifuge 5415 C) for 5 minutes to pellet bacterial cells. The supernatent was then discarded and the cell pellet resuspended in 100 μl GTE buffer (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM ethylenediamine-tetra-acetic acid (EDTA) pH 8) by pipette action or vortexing. Cells were lysed by the addition of 200 μl 0.2 M sodium hydroxide, 1 % (w/v) sodium dodecyl sulphate (SDS) and mixed by inversion. The reaction was then neutralised by the addition of 150 μl of 3 M potassium acetate, 2 M acetic acid, mixed by inversion and centrifuged at 14 000 *g* for 5 minutes to pellet cell debris (denatured proteins and chromosomal DNA). The supernatant (the cell lysate containing the renatured plasmid DNA, contaminating RNA and soluble cell fragments) was then transferred to a clean microcentrifuge tube and mixed with 800 μl isopropanol. The mixture was then centrifuged at 14 000 *g* for 10 minutes. The DNA pellet was air dried for approximately 10 minutes and resuspended in 100 μl sterile distilled water.

2.2.3.2 Precipitation of RNA

Contaminating RNA was removed by the addition of 100 μl 4 M lithium chloride, the solution was then vortexed and incubated on ice for 10 minutes. RNA was pelleted by centrifugation at 14 000 *g* for 5 minutes.

2.2.3.3 Precipitation of DNA

The plasmid DNA was precipitated by the addition of 20 µl 3 M sodium acetate, pH 4.8, 500 µl absolute ethanol (BDH) and vortexed to mix. The DNA was then pelleted by centrifugation at 14 000 *g* for 10 minutes, washed with 200 µl 70 % (w/v) ethanol and air dried. The DNA was finally resuspended in 50 µl sterile distilled water and stored at -20 °C.

2.2.4 Purification of plasmid DNA (large scale)

The Promega Wizard® Plus SV Miniprep DNA Purification System was used to prepare DNA from 10 ml cell cultures. The manufacturers' instructions were followed for this procedure. Spectrophotometer readings were made to determine the DNA concentration, and the DNA was diluted to approximately 500 ng/µl.

2.2.5 Agarose gel electrophoresis (for DNA)

Samples of DNA were mixed with 1/3 volume of Ficoll loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 15 % (w/v) Ficoll; Sambrook *et al.*, 1989).

Agarose gels were run either in a midi (60 ml) or minigel (30 ml) apparatus (Biorad™). Gels were prepared as 1 % - 1.5 % agarose dissolved in 1x TBE buffer (0.09 M Tris, 0.09 M boric acid, 0.0025 M EDTA; Sambrooke *et al.*, 1989); these concentrations of agarose were used to separate DNA fragments of between approximately 5000 and 300 base pairs (Millam 1991). Midigels and minigels were run in 1x TBE buffer at 170 V and 140 V respectively. A stock of ethidium bromide (10 mg/ml) was diluted in water at a ratio of approximately 1:20 000. Gels were then stained for 20 to 30 minutes before viewing on a longwave UV transilluminator (302 nm).

2.2.6 Spectrophotometric determination of DNA or RNA

Measurements of optical density (OD) were made using a Perkin-Elmer Lambda BIO UV/VIS spectrometer or a Beckman Du® 640 spectrophotometer. An absorbance at 260 nm provided an estimate of DNA or RNA yield; where 1 OD is equivalent to 50

µg/ml DNA and 40 µg/ml RNA. The 260/280 ratio was used to estimate the purity of the preparations, with ratios of less than 1.8 for DNA, or 2.0 for RNA suggesting possible protein contamination.

2.2.7 Sequencing reaction

DNA was sequenced using the commercial kit ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction (with AmpliTaq® DNA Polymerase, FS). A 10 µl DNA sequencing reaction was mixed on ice and contained 350 ng template DNA, 20 ng primer (3.2 pmol/µl), 4 µl terminator mix and sterile water. The thermal cycling reaction was 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes with a ramp rate of 1°C per second and repeated for 25 cycles. The reaction product was then precipitated by the addition of 10 µl sterile distilled water, 2 µl 3 M sodium acetate pH 4.6 and 50 µl of absolute ethanol. The reaction was mixed, incubated on ice for 15 minutes and then centrifuged at 14 000 *g* for 10 minutes. The DNA pellet was washed once with 70 % (v/v) ethanol and then air-dried. The DNA pellet product was electrophoresed on site using an ABI 377 automatic sequencer (performed in-house by Claire McQuade, CMG).

2.2.8 Extraction of DNA with phenol:chloroform (for protein removal) and ethanol precipitation

Protein contaminants (including modification enzymes) were extracted from DNA solutions by the addition of an equal volume of phenol:chloroform (1:1, v/v). The mixture was then vortexed and centrifuged at 14 000 *g* for 5 minutes. The supernatant (containing DNA) was then transferred to a new microcentrifuge tube. DNA was precipitated by the addition of 20 µl 3 M sodium acetate pH 4.8 and 500 µl absolute ethanol. The solution was incubated at -20 °C for 1 hour and then centrifuged at 14 000 *g* for 10 minutes. The recovered DNA pellet was then washed with 70 % ethanol, allowed to air dry and re-suspended in sterile distilled water.

2.2.9 Modification enzymes

Modification enzymes T4 DNA ligase (Gibco), T4 DNA polymerase (Gibco) and *E. coli* DNA polymerase I (Klenow fragment, a DNA-dependent DNA polymerase; Biolabs) were used with the reaction buffers recommended by the manufacturer.

2.2.10 Restriction Enzyme reactions

The reactions were prepared as directed by the manufacturer.

2.2.11 Purification of DNA fragments (by gel extraction)

DNA electropheresis was carried out in 1.2 % agarose gels (see 2.2.5) and run in 1x TBE buffer at 70 V. The DNA fragment was then excised from the agarose gel using a scalpel. Unwanted agarose was removed from the gel slice prior to weighing. DNA was extracted and purified from the agarose slice using a Qiaquick gel extraction kit (Qiagen) according to manufacturer's instructions. DNA was eluted from a spin column in 30 µl nuclease-free water.

2.2.12 Creating blunt ends on ds DNA - using Polymerase I (Klenow fragment) or T4 DNA polymerase

Digested DNA was purified from either gel fragments (2.2.11) or ethanol precipitated after a phenol:chloroform extraction (2.2.8). Termini of linerized DNA were blunted with DNA polymerase I (Klenow fragment) or T4 DNA polymerase. The reaction mixture comprised of 1 - 5 µg DNA, 5 U enzyme, 1x reaction buffer (for Klenow; Boehringer restriction enzyme Buffer L; 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithioerythritol (DTE)) and 1 mM deoxynucleoside triphosphates (dNTPs). The reaction was incubated at 37 °C for 30 minutes and then terminated by extracting the DNA from solution with phenol:chloroform (1:1, v/v). The blunt ended DNA was then ready for ligation.

2.2.13 Ligation

Slices of Low Melting Point agarose (Gibco) containing restricted vector DNA and gene fragment were melted individually at 70 °C for 10 minutes, then ligated (in a 1:2.5 ratio) in a 50 µl reaction mixture containing: 1x ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM magnesium chloride, 1mM ATP, 1 mM dithiothreitol (DTT), 5% (w/v) PEG-8000), 2 U T4 DNA ligase. The reaction was then mixed and incubated overnight at room temperature. 150 µl of TE buffer (0.5 M Tris pH 8, 1 mM EDTA) was then added to the ligation reaction and heated at 70 °C for 10 minutes. A single phenol extraction (1:1, v/v) was then carried out followed by a phenol:chloroform (1:1, v/v) extraction (2.2.8). Three µl of glycogen (1mg/ml) was then added to the supernatant and the DNA ethanol precipitated (2.2.8). The washed DNA pellet was then allowed to air dry and re-suspended in 5 µl of sterile distilled water. Half of this reaction mixture was electroporated into competent cells according to 2.2.14

2.2.14 Electroporation

The Biorad Gene Pulser II was used for all *E. coli* electro-transformations. DNA (0.5 - 2 µl) was added to 50 µl *E. coli* competent cells (DH5α) and transferred to a 0.2 cm-gap cuvette (stored on ice). Cells were electroporated at 2.5 KV and 200 Ohms resistance. One ml of SOC (20 g bacto-tryptone, 5 g bacto-yeast extract, 8.5 mM sodium chloride, 20 mM glucose, 2.5 mM potassium chlorise, 10 mM magnesium chloride at pH 7.0; Sambrooke *et al.*, 1989) was then added to the electroporated cells. Cells were incubated for 1 hour at 37 °C and then plated onto LB medium containing ampicillin 50 µg/ml. Colonies were visible after incubation at 37 °C for 6 to 8 hours.

2.2.15 Extraction and purification of total RNA from plant tissue

Two leaf discs were removed from an infected leaf using a cork borer (no. 7 or 8) and placed into a 1.5 ml microcentrifuge tube. Leaf samples were immediately frozen in liquid nitrogen (LN_2) and then ground using a plastic pestle. Samples were then vortexed with a mixture of 400 µl of TLES buffer (0.1 M lithium chloride, 100 mM Tris-HCl pH 8. 10 mM EDTA, 1 % SDS) and 400 µl phenol (pH 8) for 1 minute. The mixture was then

centrifuged at 14 000 *g* for 10 minutes to pellet cell debris. The supernatant was then transferred to a fresh tube and 200 µl of phenol and 200 µl chloroform added. The mixture was then vortexed for 30 seconds and centrifuged at 14 000 *g* for 5 minutes. This phenol:chloroform extraction step was then repeated. The RNA was then precipitated by the addition of an equal volume of 4 M lithium chloride and stored for 2 to 12 hours at -20 °C (or for long term storage). The RNA was then centrifuged at 14 000 *g* for 10 minutes. The RNA pellet was rinsed with 70 % (v/v) ethanol, air dried and resuspended in 15-20 µl sterile distilled water (based on method by Verwoerd *et al.*, 1989).

2.2.16 Reverse transcription - Polymerase Chain Reaction (RT-PCR)

2.2.16.1 First-strand cDNA synthesis

The virus RNA2 was reverse transcribed into single strand (ss) cDNA using Moloney Murine Leukemia Virus reverse transcriptase and oligonucleotide 305; which is a universal first strand cDNA primer for the 3' terminus of the tobraviruses PEBV and TRV (see Section 2.1 for primer sequence).

A 12 µl reaction mixture containing 5 µl total plant RNA and 0.5 µl oligonucleotide primer 305 (at 1 µg/µl) was heated at 70 °C for 10 minutes (Techne Progene PCR machine), centrifuged and stored on ice. The following were then added: 4 µl 1x M-MLV-RTase buffer (5x buffer; 250 mM Tris-HCl, pH 8.3, 375 mM potassium chloride, 15 mM magnesium chloride), 2 µl 0.1 M DTT, 1 µl 10 mM dNTP mix, 200 U M-MLV RTase (Life Technologies). The 20 µl reaction mixture was then incubated at 37 °C for 60 minutes and diluted by the addition of 80 µl nuclease-free water.

2.2.16.2 Polymerase chain reaction

Double stranded DNA (ds DNA) was then synthesised by PCR in a 50 µl PCR reaction containing: 1x buffer (20 mM Tris-HCl pH 8.4, 50 mM potassium chloride, 1.5 mM magnesium chloride), 0.2 mM each dNTP, 20 pmol of upstream and downstream primers and 2.5 U *Taq* polymerase (Boerhinger and Mannheim). Either 1 µl of ds DNA (2.2.16.1) or 10 ng of purified plasmid DNA were added to the reaction. To minimize mispriming, all PCR samples were placed in a pre-heated block at 85 °C (hot start).

Thermal cycling conditions were as follows: initial denaturation at 94 °C for 60 seconds (1 cycle) and subsequently denaturation at 94 °C for 10 seconds, annealing at 50 °C for 10 seconds and synthesis at 72 °C for 75 seconds repeated for 25 to 30 cycles.

2.2.17 *In vitro* synthesis of infectious transcripts

Capped RNA2 transcripts were synthesized using a commercial kit (Ambion Megascript ™ T7 kit; Ambion Inc). The virus clone was first linearized at a unique restriction site situated in the 3' polylinker region of the cloning vector (*Sma*I for TRV/K20 clones and *Spe*I for PepRSV clones).

Five µg of plasmid DNA was digested in a 15 µl reaction with 5 U *Sma*I in 1 x Buffer A (Boerhinger and Mannheim) at 25 °C for 1-2 hours. PepRSV plasmid DNA was digested with 5 U *Sal*I, 1x Buffer H (Boerhinger and Mannheim) at 37 °C. The linearized DNA was extracted with phenol:chloroform (1:1, v/v), ethanol precipitated (2.2.8) and resuspended in 10 µl sterile distilled water. Two µl (*c.* 1 µg) of the linearized DNA was then used for each *in vitro* transcription reaction.

The 20 µl transcription reaction mixture contained: 7.5 mM ATP, 7.5 mM UTP, 7.5 mM CTP, 2.5 mM GTP, 5 mM diguanosine triphosphate (Amersham Pharmacia Biotech), 1 µg linearized cDNA, 1x reaction buffer (pre-warmed at 37 °C), 2 µl T7 RNA polymerase mix. The reaction was then briefly centrifuged to collect droplets and incubated at 37 °C for 3 hours. The RNA transcript was then precipitated by addition of an equal volume of 5 M ammonium acetate, followed by incubation on ice for 15 minutes, and centrifuged at 14 000 *g* for 5 minutes. The RNA pellet was then washed with 70 % (v/v) ethanol and centrifuged at 14 000 *g* for 2 minutes. The air dried RNA pellet was then resuspended in 40 µl nuclease-free water. The RNA concentration was determined (2.2.6) and the sample examined by formaldehyde gel electrophoresis (2.2.5).

2.2.18 Formaldehyde gel electropheresis of RNA

A 2 µl RNA sample was mixed with 5 µl formamide, 2 µl formaldehyde, 1 µl 10x MOPS buffer (0.2 M MOPS (2-(N-morpholino) propanesulphonic acid) at pH 7, 0.8 M sodium acetate and 10 mM EDTA (pH 8); Sambrooke *et al.*, 1989) and 1 µl ethidium

bromide (0.25 mg/ml). Samples were heated at 65 °C for 10 minutes, cooled on ice and collected by centrifugation. Gels were prepared in A fume hood as 1 % agarose, 7.5 % (v/v) formaldehyde and 1x MOPS buffer. Minigels were generally run in 1x MOPS buffer at 60 mA for 1 hour. Ficoll sample buffer was used as tracking dye.

2.2.19 Plant inoculation

Twenty µl of the RNA2 transcription product (*c.* 30-50 µg) was mixed with 5 µl bentonite solution (in sodium phosphate, 0.1 % (w/v) bentonite) and approximately 30-100 µg total plant RNA containing RNA1. *N. benthamiana* plants were inoculated at 4 weeks old (growth stage 2 to 3 true leaves). The first true leaf was dusted with carborundum powder and spotted with 15 µl of the transcript mixture. Leaves were subsequently gently rubbed, rinsed with tap water after 1 minute and then placed in shade overnight (MacFarlane *et al.*, 1991). Leaf and root material was harvested for inoculum after 7 to 13 days. Systemic fluorescent symptoms produced by TRV-GFP were visualized under UV light. For western blotting, tissue samples were homogenised in 1x phospho buffered saline PBS (1 ml/g). For use as future inoculum, tissue samples were homogenised in tap water, dispensed in small aliquots and frozen at -20 °C.

2.2.20 Detection and analysis of expressed proteins by Western blotting

2.2.20.1 SDS-polyacylamide gels

Gels were cast using the Mini-Protean II Electrophoresis Cell (Bio-Rad) system. A discontinuous polyacrylamide gel system was used with a 4 % Stacking gel (1 ml 40 % Acrylamide:Bis (37.5: 1; BDH), 1.26 ml 1 M Tris pH 6.8, 250 µl 10 % SDS, 7.26 ml sterile distilled water, 10 µl Tetramethyl-ethylenediamine (TEMED) and 50 µl Ammonium persulphate (APS) and a 12.5 % Resolving gel (6.25 ml 40 % Acrylamide:Bis (37.5: 1), 7.5 ml 1 M Tris pH 8.7, 100 µl 10 % SDS, 5.95 ml sterile distilled water, 5 µl TEMED and 50 µl APS).

Tissue samples were prepared by adding an equal volume of sample homogenate to Laemmli sample loading buffer (62.5 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol and 0.001 % bromophenol blue, Laemmli 1970). The sample was

then heated at 100 °C for 5 minutes and centrifuged at 14 000 *g* for 5 minutes. Aliquots of each sample were then loaded onto the gel. Proteins present in the sap samples were separated by electrophoresis in Laemmli running buffer (38 mM glycine, 50 mM Tris, 1 % SDS) and run at 100 V for 1 - 1.5 hours.

2.2.20.2 Electroblotting - protein transfer

After electrophoresis the separated proteins were transferred to a nitrocellulose membrane. The resolving gel was moved into the transfer apparatus in a series of layers: negative electrode, porous pad, filter paper, Resolving gel, reinforced nitrocellulose (Optitran BA-S 85, Schleider and Schinell), filter paper, porous pad and positive electrode. Protein transfer was then carried out in Laemmli transfer buffer (19 mM glycine, 25 mM Tris, 20 % absolute ethanol) at 100 V for 1 hour.

2.2.20.3 Antibody binding

To saturate protein binding sites, nitrocellulose filters were incubated for 30 to 60 minutes in TBS-T Buffer (10 mM Tris pH 8, 150 mM sodium chloride, 0.05% Tween 20 (SIGMA) containing 5 % nonfat dried milk. The filters were then incubated with a primary antibody (directed to target antigen, for details see Table 2.1) and TBS-T/milk powder, overnight at room temperature or 4 °C. Filters were then rinsed in TBS-T three times for 5 minutes to remove unbound antibodies and probed with a secondary antibody (IgG alkaline phosphate conjugate, anti-rabbit raised in goat; Sigma) at a 1:30 000 dilution in TBS-T/milk powder. Filters were then incubated at room temperature for 1.5 hours and subsequently rinsed three times in TBS-T for 5 minutes. Excess buffer was drained from the filter before adding a colour development substrate. An additional 10 minute wash was included if chemiluminescent substrates were to be used.

2.2.21 Chromogenic substrate

For signal detection 4 µl nitroblue tetrazolium (0.05 g dissolved in 70 % dimethylformide (DMF)) and 4 µl 5- Bromo-6-chloro-3- indolyl-phostphate (0.05 g dissolved in 100 % DMF) were added per 1 ml DEA buffer (100 mM sodium chloride, 5

mM magnesium chloride and 100 mM Tris-Cl pH 9.5). Colour development was terminated by dilution with tap water.

Table 2.1 Primary antibodies used in this study.

Primary antibody	Working dilution	Source
Anti-GNA2	1:10 000	Dr Anghared Gatehouse, Durham University
Anti-TRV	1:10 000	Dr Stuart MacFarlane, (S.C.R.I.)
Anti-GFP	1:10 000	Dr Simon Sant-Cruz (S.C.R.I)
Anti-CAM (for PepRSV)	1: 500	Dr David J. Robinson, (S.C.R.I.)

2.2.22 Chemiluminescent substrates

Two chemiluminscent substrates were used; pre-prepared CDP-*Star* detection reagent (Amersham Pharmacia Biotech), or, CDP-star® chemiluminescent substrate (Promega) was prepared by diluting the 25 mM stock solution at 1:100 with buffer (0.1 M diethanolamine, 1 mM magnesium chloride at pH 9.8).

The chemiluminescent substrate was dispersed over the upper surface of each filter and incubated for 5 minutes between two sheets of plastic. Excess substrate was removed and the filters exposed to Super RX medical Fuji x-ray film (100 NIF) and developed manually or automatically using a Compact X4 film processor (Xograph Imaging Systems). Exposure time was determined empirically and generally ranged from 5 to 60 minutes.

2.2.23 Two dimension electrophoresis, non-denaturing polyacrylamide gels

2.2.23.1 First dimension, isoelectric focusing

Isoelectric focusing was used to separate proteins present in tissue extracts based on the difference in their net charge. To study proteins in their native form, a non-denaturing environment was produced by excluding SDS from all gel mixtures and buffers. Isoelectric focusing (IEF) columns were set up in small diameter, 18 cm glass tubes. Ten ml of tube gel mix contained 10 % NP-40 (detergent), 1.33 ml IEF acrylamide solution (28.38 % acrylamide (w/v) and 1.62 % bis-acrylamide), 0.4 ml ampholines 0.08 % pH 5 - 7, 0.1 ml ampholines 0.02 % pH 3.5 – 10, 2 ml distilled water, 10 µl 10 % APS, 5.5 g urea and 7 µl TEMED. Tubes were filled with gel mix to a height of exactly 15 cm. To aid polymerization 20 µl of 8 M urea gel was overlayed onto the tube gel surface. After gel polymerization the gel overlay was replaced by 20 µl of lysis buffer (9.5 M urea, 2 % NP-40, 5 % 2-mercaptoethanol, 2 % ampholines (1.6 % pH 5-7 + 0.4 % 3.5 - 10). The lysis buffer was then overlayed with 20 µl of distilled water. A pH gradient was established through the gels by allowing ampholines to prefocus by diffusion for 30 minutes. The lysis buffer and water were then removed and replaced with 20 µl of fresh lysis buffer. The glass tubes were then transferred to a tank system containing anode buffer (0.01 M phosphoric acid H_3PO_4). The remaining space in the tube gel was carefully overlayed with cathode buffer (0.02 M sodium hydroxide) before attachment to an upper reservoir containing cathode buffer. The gels were prefocused at 200 V for 15 minutes, 300 V for 30 minutes and 400 V for 30 minutes.

The tube gels were then rinsed with distilled water to remove traces of buffer. Fifty µl root or leaf tissue sample in PBS buffer were then heated according to method 2.2.20.1, and then loaded onto the top of the tube gel and then overlayed with sample overlay buffer (9 M urea, 1 % ampholines (0.08 % pH 5 - 7 and 0.02 % pH 3.5 - 10). The tubes were then filled with cathode buffer and the top tank filled with cathode buffer. To separate the individual proteins by their isoelectric points, the gels were then run at 400 V for 12 to 20 hours. After electrophoresis the gels were removed from the tubes by rimming. The tube gels was then soaked in 10 ml equilibration buffer (10 % glycerol, 5 % mercaptoethanol, 10 % stacking buffer (see following section) for 30 minutes at room temperature.

Equilibration was then repeated with fresh buffer for a further 30 minutes. Gels were then stored in equilibration buffer at -70 °C prior to further use.

2.2.23.2 Second dimension

To separate the tissue extract proteins by molecular weight, the proteins contained in the IEF column were then resolved in a discontinuous polyacrylamide gel system. SDS was excluded from all gels and buffers. A 4.6 % Stacking gel was prepared as 6.96 ml Acryl 30 %, 5.625 ml resolving buffer (0.48 mM HCl, 3 M TRIS) 31.95 ml sterile water, 900 µl TEMED and 67.5 µl 10 % APS. A 10 % Resolving gel was prepared as 27 ml Acryl 30 %, 30 ml resolving buffer (as above), 22.8 ml sterile water, 267 µl TEMED and 800 µl 10 % APS.

The IEF gel columns were thawed and fixed to the top of the stacking gel with agarose. A well for the protein marker was made by positioning a blunt tip at one end of the gel column. Gels were run in Reservoir buffer (8 mM Tris, 0.19 M glycine). Twelve µl of Seeblue pre-stained marker (Novex) was loaded into the first well and a layer of laemmli buffer layered on the column surface to act as tracking dye. Electrophoresis was carried out at 150 - 160 V overnight.

2.2.23.3 Electroblotting

The stacking gel was removed and the resolving gel soaked in transfer buffer to remove traces of mercaptoethanol. The transfer was carried out as described in section 2.2.20.2. The nitrocellulose membrane (Hybond - C pure nitrocellulose membrane, Amersham Life Science) was soaked in transfer buffer prior to use. Air bubbles were removed by rolling a glass rod over the uppermost layer of filter paper. Protein transfer was carried out in Transfer buffer (0.02 M Tris, 0.19 M glycine, 20 % (v/v) methanol and sterile distilled water) by electroblotting. To visualize the proteins the filters were blocked and probed with primary and secondary antibodies according to section 2.2.20.3.

2.2.24 Plant tissue print

Tissue printing methods were adapted from Mansky *et al.*, (1990). Root or leaf materials were laid onto dry nitrocellulose. A second nitrocellulose layer was then overlaid onto the plant tissue and held securely. The nitrocellulose and plant tissue was then covered by a plastic sheet and squashed using a cylinder. The resulting tissue blots were then soaked in TBS-T for 20 minutes and the remaining fragments of plant tissue removed. The tissue blots were then incubated in TBST/milk powder (2.2.20.3) for 30 to 60 minutes, and probed with primary and secondary antibodies according to the methods outlined in 2.2.20.3.

2.3 Construction of the TRV snowdrop gene expression vector, TRV-GNA

2.3.1 Subcloning of the GNA sequence into the PT7Blue cloning vector

A cDNA coding for the snowdrop lectin '*Galanthus nivalis* agglutinin' (LecGNA2 accession number M55556) was amplified by polymerase chain reaction (PCR) from the plasmid p1GNA2 (kindly supplied by Axis Genetics Inc.). PCR mutagenesis was used to engineer specific alterations at the 5' and 3' termini of the GNA2 gene. An unique restriction site for *Avr*II was introduced immediately after the initiation codon (ATG) at position 21 in the published sequence, and a unique *Kpn*I site was introduced down stream of the GNA2 termination codon at position 498 (illustrated in Figure 2.1 A). The changes introduced into the sequence were designed to enable direct insertion of the GNA gene into the TRV gene expression vector (directional cloning). The sequence of the oligonucleotide primers used to amplify the GNA coding region were as follows;

N-terminus: 5' CAAA**ATG**CCTAGGGCAAGTCTC 3' [Insert: start codon (bold) and a *Avr*II site (underlined)]

C-terminus: 5' AGTTAAAAG[illegible] GG**TCA**TTAC 3' [Insert: termination codon (bold) and a *Kpn*I site (underlined)]

The 50 µl PCR reaction contained 1 µl of 50 ng/µl DNA template (p1GNA2), 1x PCR buffer (Boehringer and Mannheim), 20 pmol of each primer, 5 µl of 2 mM dNTPs mix and 5 U *Taq* polymerase. All reagents were kept on ice prior to PCR and the samples were placed in a pre-heated PCR block to prevent mis-priming. The following thermal cycling reaction was used to amplify the GNA2 sequence; I) 1 cycle at 94 °C for 60 seconds; II) 3 cycles of 94 °C for 10 seconds, 50 °C for 20 seconds and 68 °C for 45 seconds; III) 25 cycles of 94 °C for 10 seconds, 60 °C for 20 seconds and 68 °C for 45 seconds; IV) 1 cycle of 8 °C for 10 seconds.

Ten µl PCR product and 4 µl Ficoll were loaded into a 1.2 % agarose minigel and electrophoresed at 140 V (2.2.5). A 498 bp DNA fragment correlating to the GNA gene was observed under UV light after staining with Ethidium bromide.

The GNA PCR product was ligated (2.2.13) into pT7Blue T-Vector (Novagen) using T4 DNA ligase. The re-circularised DNA was then introduced into competent *E coli* DH5α cells by electroporation (2.2.14). Recombinant plasmids were identified by blue-white screening after growth on isopropyl-β-D-thiogalactopyranoside / 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ampicillin (IPTG/X-gal/amp) medium. Twelve transformant clones were chosen for further characterization. Individual, white colonies were selected from the culture plate and bacterial cultures prepared in LB/ampicillin selection medium according to method 2.2.2. Alkaline lysis was then carried out to isolate plasmid DNA from each clone (2.2.3).

To confirm that a full-length GNA sequence was subcloned into the plasmid DNA, the recombinant plasmid DNA was first screened by restriction endonuclease analysis. A 20 µl double digestion reaction mixture containing 0.5 - 2 µl c. 250 ng of plasmid DNA, 1x Buffer (NEB4), 2.5 U *Sac*I (Biolabs) and 2.5 U *Xba*I (Roche) and 1x BSA (100 µg/ml) (Biolabs). Then to determine the orientation of the GNA sequence relative to the vector a 20 µl digestion reaction containing either i) 0.5 µl (5 U) of *Eco*RI and 1x Buffer (H) (Boehringer and Mannheim), or, ii) *Hind*III and 1x Buffer (B) (Promega) were carried out. All digestion reactions were then incubated at 37 °C for 1 to 2 hours and terminated by heat inactivation. The products of the restriction digestions were then characterized by gel analysis. PT7Blue/GNA clone 7 was subsequently selected for further analysis as this

clone produced the expected bands after digestion (see Figure 2.2 for a schematic representation of the TRV-GFP cloning process. The nucleotide sequence of the cloned PCR-amplified GNA gene was then determined following the procedure outlined in method 2.2.7 using oligonucleotide 250.

Sequence alignment indicated that two base changes had occured at two positions from the published sequence. The complete GNA sequence is presented in Figure 2.1 A) and differs at two positions from the published sequence. The first base substitution from gtg to gcg occurs at the start of the mature peptide sequence (at position 86) and would result in the incorporation of alanine rather than valine. However, as both amino acids have nonpolar side chains, which characteristically cluster on the inside of a protein (Alberts *et al.*, 1989), it was thought that the GNA protein structure would not be significantly altered. The second base substitution from gta to gaa occurs in the C-terminal part of the protein peptide (at position 489) and would result in the incorporation of glutamic acid (acidic side chain) rather than valine (nonpolar). However, as the C-terminal peptide of GNA is removed postranslationally (Van Damme and Peumans,1996) this substitution should not affect the structure of the mature protein.

2.3.2 Subcloning of the GNA gene into the TRV gene expression vector

The TRV virus had been engineered to function as a gene expression vector by Stuart MacFarlane. Plasmid pK20GFPb carries TRV RNA2, which expresses the green fluorescent protein (GFP) from a duplicated virus promoter; GFP expression by this clone is described in greater detail in Chapter 3. In the present study, the GNA gene isolated in section 2.3.1 was substituted for pK20GFPb's GFP sequence. The GFP sequence was removed from the pK20GFPb plasmid by digestion with *Nhe*I (position 4 after the GFP initiation codon) and *Kpn*I (downstream of the GFP termination codon). A 15 µl digestion reaction mixture was prepared containing c. 2 µg of pK20GFPb plasmid DNA, 1x Buffer NEB2, 1x BSA (100 µg/ml), 2.5 U *Nhe*I (Biolabs) and 2 U *Kpn*I (Gibco). The reaction was incubated at 37 °C for 1 to 2 hours and then stopped by heat inactivation of the enzymes at 65 °C for 20 minutes.

The GNA sequence was then cleaved from c. 2 µg pT7Blue/GNA clone 7 by digestion with 2 U *Avr*II (Biolabs) and 2 U *Kpn*I in a 15 µl digestion reaction mixture containing 1x Buffer NEB1 and 1x BSA (100 µg/ml). The mixture was then incubated at 37 °C for 1 hour. The digestion products for each 15 µl reaction were separated on a 1 % w/v Low Melting Point agarose gel (electrophoresis method 2.2.5). The DNA fragments correlating to the GNA gene fragment (approximately 500 bp) and pK20TRV/ΔGFP vector were excised from the gel and melted at 70 °C for 10 minutes prior to ligation following method 2.2.13. Plasmid DNA from a TRV-GNA clone was re-transformed into *E. coli* cells according to method 2.2.13 and incubated over-night on ampicillin selection plates. A bacterial colony was then grown overnight in medium and a glycerol stock prepared (2.2.2.2). A schematic representation of the resulting clone TRV-GNA is shown in Figure 2.2 F.

2.3.4 Production of viral inoculum by *in vitro* transcription

Approximately 4-5 µg of SV miniprep plasmid DNA was linearized by digestion with 15 U *Sma*I in a 15 µl reaction with 1x Buffer A for 1-2 hours. The linearized plasmid DNA was then phenol extracted, ethanol precipitated and resuspended in 10 µl sterile distilled water following the method in section 2.2.8. Two µl (c. 1 µg) of the linearized DNA plasmid was then used for a single transcription reaction (2.2.17).

Four week old *N. benthamiana* plants were then mechanically inoculated with a mixture of total RNA from plants infected with an NM isolate of TRV PpK20 and transcripts of TRV RNA2 expressing GFP or GNA. Leaf and root material was harvested after a period of 7 to 10 days. The tissue was processed in 1x PBS and then stored at -20 °C. Western blot analysis was used to confirm the presence of coat protein or GNA following the method outlined in 2.2.20. Leaf and root blots for the first set of TRV-GFP transcript inoculated plants probed with GNA antiserum are presented in Chapter 4. The method described above was used as a standard protocol throughout the research project.

2.4 Production of two TRV virus mutant clones

2.4.1 A mutant TRV-GNA clone

The TRV-GNA clone was manipulated by simultaneously treating the plasmid DNA with the endonuclease *Sph*I, which cuts at position 254 in the GNA gene, and Klenow polymerase to produce a frameshift mutation in the GNA gene. This clone would be used in subsequent bioassay experiments as a 'GNA-control' treatment against which the effects of GNA could be compared.

Manipulation of the TRV-GNA plasmid DNA (c. 2 μg) was carried out in a single reaction. A 20 μl reaction mixture containing 10 U *Sph*I (Biolabs), 1x Buffer NEB2, 5 U Klenow polymerase and 0.5 μl of 2 mM dNTPs was incubated at 37 °C for 1 hour. The enzymes were then inactivated by heating at 70 °C for 10 minutes. The DNA was then ethanol precipitated (2.2.8) and the DNA pellet resuspended in 10 μl sterile distilled water. Approximately 200 ng of *Sph*I-blunt plasmid DNA was then re-ligated in 1x ligase buffer with 1 U T4 DNA ligase. The reaction was incubated at room temperature overnight (approximately 12 hours). A 3 μl volume of the ligation mixture was then directly electroporated into competent *E. coli* DH5α cells (2.2.14).

Recombinant plasmid DNA was isolated from six independent colonies, cultured according to method 2.2.2.1 and the DNA purified using a Promega kit (2.2.4). To characterize the transformants 200 ng of plasmid DNA (350 ng for multiple digest) was then incubated in a 15 μl reaction mix containing either: i) 2.5 U *Sph*I (located at position 254 in the GNA gene) and 1x Buffer M, or ii) 2.5 U *Sph*I + *Bgl*II (located in the vector at position 650 and 1200) and 1x Buffer H, and then incubated at 37 °C for 1 hour. The digestion products were then electrophoresed on a 1 % agarose gel and the results analysed. Clones carrying a mutation at the *Sph*I site would no longer be cut by this enzyme and thus produce a band of 550 bp correlating to digestion at the two *Bgl*II sites (position 650 and 1200 in TRV RNA2). Had the *Sph*I site cut then an additional band of 910 bp would have been detected.

Two clones were identified which cleaved at the *Bgl*II sites only. A 10 μl sequencing reaction with 20 pmol oligonucleotide 250 (primer for the subgenomic promoter derived

Figure 2.1 A) Nucleotide coding sequence for the common snowdrop lectin '*Galanthus nivalis* agglutinin' (LECGNA 2). Positions of each oligonucleotide primer are indicated below and amplify a 498 bp GNA gene fragment that was subsequently cloned into the cloning vector pT7Blue. Base pair differences are indicated in green. Cloning sites incorporated into the sequence are shown as cctagg and , *Avr*II site *Kpn*I site respectively. **Atg** start codon for the signal peptide. The mature peptide is coded from position 87 ( ) to position 401 ( ), the beginning of the C-terminal peptide and is the unique *Sph*I restriction site (position 254). B) Frameshift mutation detected in GNA after sequencing TRV-fsGNA clone 5. Nucleotide sequence of GNA (black sequence) and fsGNA (blue sequence) are aligned to indicate the position of the *Sph*I site (), base pair deletions ( ) and the presense of a premature termination codon (att) at position 333.

A) Nucleotide sequence of GNA

1 Caactacaag ttacaaa**atg** gctaaggcaa gtctcctcat tttggccgcc atcttccttg

5' primer caaa**atg**cctagggcaagtctc

61 gtgtcatcac accatcttgc ctgagt gaca atattttgta ctccggtgag actctctcta

121 caggggaatt tctcaactac ggaattteg ttttatcat gcaagaggac tgcaatctgg

181 tcttgtacga cgtggacaag ccaatctggg caacaaacac aggtggtctc tcccgtagct

241 gcttcctca agact gatgggaacc tcgtggtgta caacccatcg aacaaaccga

301 tttgggcaag caacactgga ggccaaaatg ggaattacgt gtgcatccta cagaaggata

361 ggaatgttgt gatctacgga actgatcgtt gggctactgg a actcacacc ggacttgttg

421 gaattcccgc atcgccaccc tcagagaaat atcctactgc tggaaagata aagcttgtga

481 cggcaaagt a a**tga**ccggtg atcttttaac ttgcatgtat gtgggaagag taataaaata

cat **tact**ggc gaaaattga 3' primer

541 agtgcatttg agataatcga cctcgtcgcg

B) Nucleotide sequence of frameshift GNA mutant

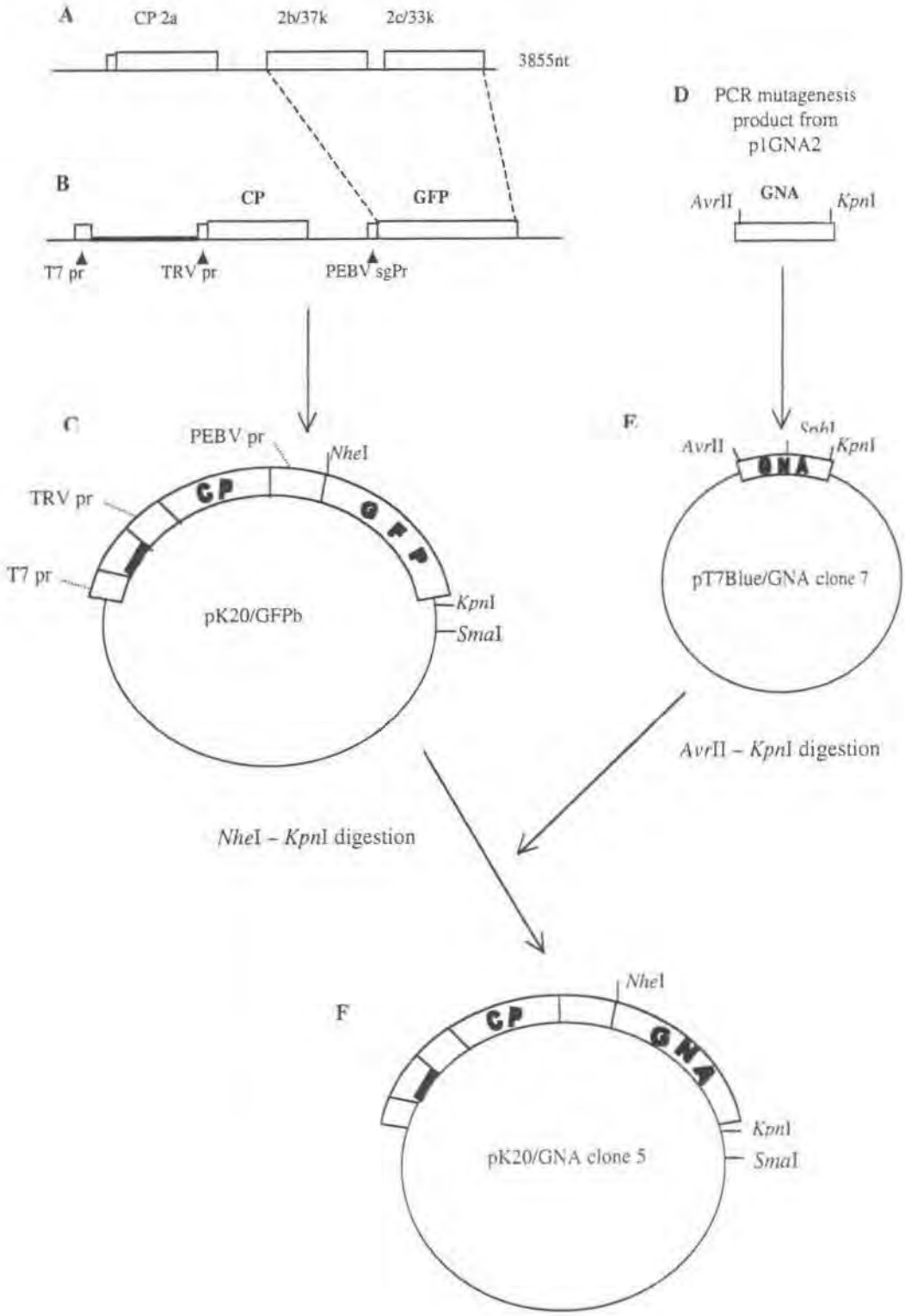
241 gcttcctca agact gatgggaacc tcgtggtgta caacccatcg aacaaaccga

gcttcctca cact gatgggaacc gatgggaacc caacccatcg aacaaaccga

301 tttgggcaag caacactgga ggccaaaatg ggaattacgt gtgcatccta cagaaggata

tttgggcaag caacactgga ggccaaaatg ggaatt

Figure 2.2 Cloning history of TRV PpK20 virus expression vector pTRV/GFPb (C) from the native TRV RNA2 molecule (A) and replacement of 2b and 2c genes with GFP linked to a PEBV sub genomic promoter (sgP) (B). Cloning strategy of pK20/GNA clone 5 (F) from PCR product (D). into the cloning vector pT7Blue/GNA (E) and virus expression vector (F) (MacFarlane and Popovich, 2000).



from PEBV/A56 RNA2) was then prepared for each *Sph*I-frameshift TRV-fsGNA clone and the GNA gene sequenced following method 2.2.7. (see section 2.1 for oligonucleotide sequence). Nucleic acid sequence comparison indicated that the deletion of 7 bp at position 250 to 256 in the GNA sequence of pK20/fsGNA clone 5 (TRV-fsGNA) would result in a frameshift in the codon reading frame after this position. Whereas, the 6 bp deletion at position 249 to 254 in pK20/fsGNA clone 2 would not. The nucleotide sequence of the mutant GNA clone 5 is presented in Figure 2.1 B).

The protocol described in section 2.2.17 was followed to produce capped RNA2 transcript from *Sma*I-linearized TRV-fsGNA plasmid DNA. Six 4 week old *N. benthamiana* plants were then inoculated with TRV RNA1 and the TRV-fsGNA transcipt and the leaf tissues harvested at 7 - 10 dpi. The presence of the TRV-fsGNA RNA and the frameshift GNA gene was verified in systemic infections by RT-PCR, since anti-fsGNA antibodies were not available. Reverse transcription of TRV RNA was carried out using primer 305. PCR amplification of a portion of TRV RNA2 carrying the GNA gene used primers 250 and 166 (the latter is specific to the 3' terminal region of pK20). The reaction was prepared according to that described in 2.2.16. The amplification conditions used were as follows; 94 °C for 60 seconds (1 cycle), and 94 °C for 10 seconds followed by 45 °C for 20 seconds and 72 °C for 60 seconds repeated for 30 cycles. Ten µl of PCR product amplified from each of the inoculated plants was run on a 1 % agarose gel at 140 V. TRV-fsGNA RNA2 was detected in 5 of the 6 inoculated plants. The presence of the GNA gene was then confirmed by single digestions with *Sph*I, *Hind*III and *Bsr*GI which indicated that an intact full length GNA gene was present in the TRV-fsGNA RNA2 molecule and that the *Sph*I-site mutation was maintained, indicating that the mutation was stably maintained. Western blot analysis was routinely used to detect virus coat protein in systemic infections after inoculation with transcript (first passage) or sap (second passage) to *N. benthamiana* plants.

A BLAST database search at w.w.w.nptl.gov (Altshul *et al.*, 1997) was carried out for the frame-shift GNA nucleotide sequence between position 249 and 570. Analysis by sequence alignment indicated that the nearest match for the frameshift amino acid sequence had less than 37 % identity to a hypothetical *Caenorhabditis elegans* (nematode)

protein Y25C1A.3, followed by a hypothetical glycine-rich protein isolated from Mesorhi (ref NP 102359.1). No match for the frameshift GNA amino acid sequence suggested that nonsense amino acid coding was translated after the frameshift.

2.4.2 A GFP deletion mutant

A GFP-deleted TRV RNA2 (TRV-ΔGFP clone) was created to provide a 'virus-only' control treatment for the subsequent bioassays experiments.

The GFP sequence was deleted from the pK20GFPb plasmid following digestion with *Nhe*I and *Kpn*I. The digested pK20/ΔGFP vector DNA was separated from the GFP gene by electrophoresis on 1 % agarose and isolated and processed using a Qiaquick gel extraction kit (Qiagen 2.2.11). The purified DNA was treated with Klenow fragment (polymerase I) to blunt the single stranded overhanging termini. The reaction was terminated by a single phenol:chloroform extraction. The linearized pK20/ΔGFP fragment was then re-ligated with 1 U T4 DNA mixture ligase in 1x ligase buffer and the reaction incubated at room temperature overnight (approximately 12 hours). A 2 µl volume of the ligation was then electroporated into competent *E. coli* DH5α cells (2.2.14).

Plasmid DNA was then isolated from several transformant clones by a miniprep kit (Promega, 2.2.4). To confirm the deletion of the GFP gene, a series of restriction digests was carried out including; i) *Bgl*II (1x Buffer NEB3), ii) *Sna*BI (1x Buffer NEB4), iii) *Hind*III (1x Buffer B), iv) *Nco*I (1x Buffer NEB4), v) *Kpn*I (1x Buffer REact® 4 and 1x BSA (100 µg/ml)), and vi) *Bgl*II and *Sna*BII (1x Buffer NEB4 and 1x BSA (100 µg/ml)). Fifteen µl of digested DNA was then electrophoresed on a 1 % agarose gel and the pattern of the restriction digests analysed. Two pK20/ΔGFP clones (TRV-ΔGFP clone 1 and 2) were identified and re-transformed into competent DH5α cells.

The protocol described in section 2.2.17 was followed to produce *Sma*I linearized TRV-ΔGFP plasmid DNA. Capped RNA2 transcripts were then produced for inoculation to *N. benthamiana* plants. Total plant RNA was then extracted from leaf tissue samples harvested at 10 dpi, and RT-PCR was used to detect the TRV-ΔGFP RNA2. First strand cDNA synthesis was then reverse transcribed from the virus RNA2 using oligonucleotide 305 following the method described in 2.2.16.1. The full-length TRV-ΔGFP clone was

synthesised using oligonucleotide 254 (specific to the CP gene at position 625-643) and oligonucleotide 300 (specific to the 3' non-coding region position 3534 - 3555) (see section 2.1 for oligonucleotide sequence). The reaction was prepared according to that described in 2.2.16. The amplification conditions used were as follows; 94 °C for 60 seconds (1 cycle), and 94 °C for 10 seconds followed by 53 °C for 30 seconds and 72 °C for 90 seconds repeated for 25 cycles. Ten µl of PCR product amplified from each of the inoculated plants were then run on a 1 % agarose gel at 140 V. Gel analysis indicated that both recombinant clones tested were TRV-ΔGFP. TRV-ΔGFP inoculum was routinely produced following the protocol described for TRV-GNA. Western blot analysis was used to detect the virus coat protein in TRV-ΔGFP transcript-inoculated and sap-inoculated *N. benthamiana* plants.

2.5 Molecular cloning of *Pepper Ringspot Virus*

A second virus expression vector based on the *PepperRingspot Virus* (PepRSV) (obtained from Stuart Macfarlane, SCRI) was chosen to complement the studies carried out with TRV. It was hoped that the differences in gene expression level of these two viruses might provide an alternative system for screening pesticidal gene products, particularly if low concentrations of the active ingredient were an important factor in their antifeedant property. PT7Blue/PepRSV-GFP clone 15 was modified to express GNA. This was achieved following digestion with *Nhe*I and *Cla*I that flank the GFP sequence. The overhanging termini of the digested PepRSV vector would then be compatible with the GNA fragment that was excised from pT7Blue/GNA clone 7 by digestion with *Avr*II and *Acc*I.

2.5.1 Construction of the PepRSV-GNA vector

Approximately 12.5 µg of PepRSV-GFP and 1.6 µg of pT7Blue/GNA was linearized with 0.75 µl of *Nhe*I/*Cla*I and *Avr*II/*Acc*I respectively, and incubated for 2 hours at 37 °C. The digestion products were then separated on a 1 % low melting agarose at 60 V for 2 hours. The DNA bands correlating to the GNA fragment (approximately 500 bp) and the PepRSV/ΔGFP vector (5 kb) were then excised from the gel and ligated together

using T4 DNA ligase in a 1: 2.5, vector: fragment ratio following the procedure in section 2.2.13. After electroporation ten transformed colonies were selected, cultured overnight in selection medium (LB Amp) and the plasmid DNA isolated according to method 2.2.3.1.

The recombinant plasmids were then characterized with a series of restriction digestions. An *Eco*RI restriction digest was initially carried out to confirm the cloning of the GNA sequence into the vector (position 592 in vector, position 421 in GNA and position 750 in GFP). A series of single digestions of *Eco*RI, *Ssp*I, *Hind*III, *Pst*I, *Pfl*MI and *Sac*I were then undertaken to confirm the identity of potential PepRSV-GNA recombinant plasmids. PepRSV-GNA clone 9 produced the expected digestion products and was re-transformed into competent DH5α cells. A schematic representation of the PepRSV-GNA clone 9 is shown below in Figure 2.2.

2.5.2 Production of two PepRSV virus mutant clones

2.5.2.1 A mutant PepRSV-GNA clone

The aim of this mutant was to provide a 'GNA-control' treatment for subsequent bioassays that would be comparable to the *Sph*I derived TRV-fsGNA mutant. However, in the PT7Blue vector an additional *Sph*I site was also present in the 3' linker region at postion 55. Partial restriction was achieved by preparing a series of digestion reactions that contained 663 ng of DNA, 1x buffer and either 0.25, 0.5, 1.0, 1.5 or 2.0 U of *Sph*I enzyme. The reaction was then incubated for only 15 minutes at 37 °C, held on ice and dilution with 85 µl sterile distilled water added. A single phenol:chloroform extraction was then carried out to terminate the digestion, the plasmid DNA ethanol precipitated and re-suspended in 15 µl sterile distilled water. The digestion products were then resolved on 1 % agarose. Vector fragments digested at one or both *Sph*I sites were then distinguished by size. Larger vector fragments (single site digested) were then excised, combined and weighed. The DNA was extracted from the agarose gel using the Qiagen gel-extraction kit (2.2.11) and eluted in 30 µl of sterile distilled water.

Approximately 100 ng of *Sph*I linearized DNA was blunted in a 35 µl reaction containing 5 U Klenow fragment, 0.9 µl of 2 mM dNTPs and 1x buffer. The reaction was incubated at 37 °C for 15 minutes and then heated at 70 °C for 10 minutes. Ten µl of the

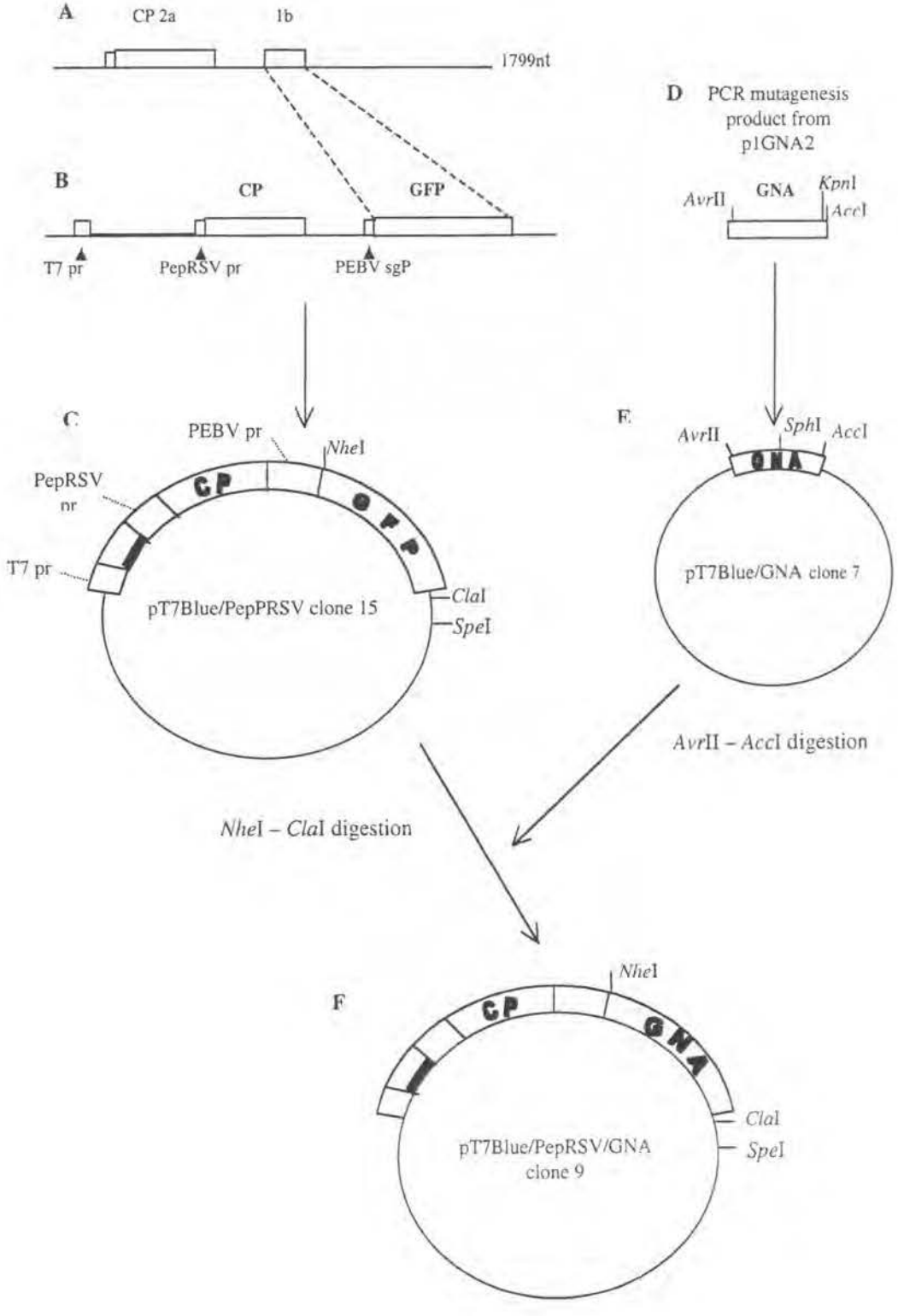
blunted plasmid DNA was then ligated in a 15 µl reaction containing 1x buffer and 1 U T4 ligase and incubated overnight at room temperature. Aliquots of the ligation mixture were then electroporated into Top10 competent *E. coli* cells. Two transformants were characterized by double digestion with: i) *Bam*HI + *Sal*I, 1x Buffer NEB3 and 1x BSA (100 µg/ml), ii) *Sph*I + *Sac*I and1x Buffer NEB4 and iii) *Pst*I + *Sty*I, 1x Buffer NEB3 and 1x BSA (100 µg/ml). Gel analysis indicated that PepRSV/fsGNA clone A was not digested with *Sph*I. The plasmid DNA was then re-transformed into competent Top10 cells.

2.5.2.3 GFP deletion mutant

A PepRSV-GNA-deletion was created for this study by creating a frameshift at a unique *Sty*I site at position 56 in the signal peptide region of the GNA sequence. The aim of this treatment would be premature termination of GNA translation or the production of nonsence coding. This virus mutant would be used as the 'virus-only' treatment for the subsquent bioassays experiments.

A 15 µl reaction containing approximately 664 ng PepRSV-GNA clone 9 plasmid DNA, 1x Buffer NEB3 and 0.5 µl *Sty*I was incubated at 37 °C for 2 hours. A single phenol:chloroform extraction step followed by ethanol precipitation (2.2.8) was carried out to terminate the reaction and the pellet washed and resuspended in 10 µl sterile distilled water. Gel analysis was then used to confirm linearization of the vector plasmid. Five µl of *Sty*I linearised DNA was then blunted in a 10 µl reaction containing 0.5 µl T4 DNA polymerase, 1x polymerase buffer, 1 mM dNTPs. The reaction was then incubated at 37 °C for 15 minutes and inactivated at 70 °C for 10 minutes. A 15 µl ligation reaction was then prepared with 5 µl of blunted DNA, 1x buffer and 1 U T4 ligase and incubated at room temperature overnight. A 2.5 µl aliquot of the ligation mixure was then transformed into competent cells and screened on antibiotic selection plates. Plasmid DNA was isolated from 9 colonies. Recombinant *Sty*I frameshift mutant plasmids were characterized after digestion with *Sty*I or *Afl*III. Five colonies were then analyzed further by double digestion with i) *Bam*HI + *Sal*I, ii) *Sph*I + *Sac*I and iii) *Pst*I + *Sty*I. Gel analysis indicated that PepRSV-ΔGNA clone 4 was not digested by *Sty*I, which indicated that for this clone, the

Figure 2.3 Cloning history of PepRSV expression vector pT7Blue/PepRSV/GFP clone 15 from the PepRSV CAM RNA2 molecule (A) and replacement of a partial copy of the RNA 1b gene with GFP linked to a subgenomic PEBV promoter (sgP) (B). Cloning strategy of PepRSV/GNAclone 9 (F) from the cloning vector pT7Blue/GNA (E) to the virus expression vector (F). (MacFarlane and Popovich 2000).



*Sty*I was unrecognizable and therefore mutated. The plasmid DNA was then re-transformed into competent Top10 cells.

2.5.2.4 Production of viral inoculum by *in vitro* transcription

Each of the viral RNA2 clones was linearized by the restriction enzyme *Spe*1 according to method 2.2.17 and the RNA2 transcript prepared. The mass yield of RNA2 was then spectrophotometrically quantified.

Chapter 3

Expression of green fluorescent protein by the *Tobacco Rattle Virus* vector

3.1 Introduction

3.1.1 Green fluorescent protein

The green fluorescent protein (GFP) originates from the jellyfish *Aequorea victoria. In vivo* the GFP protein is the final stage of an energy transfer pathway which results in the visible bioluminescence of intact jellyfish tissue (Morin and Hastings, 1971). The primary fluorophore in this pathway is aequorin that emits blue light at 470 nm, which is close to the excitation peak of the GFP protein, GFP then converts the blue fluorescence into green bioluminescence at 508 nm (Tsien 1998). Ultra violet light (UV, 470 nm) lamps, blue light and sunlight induce GFP fluorescence (Tsien 1998, An 1985). The green fluorescent protein has been recently reviewed by Tsien (1998).

3.1.2 GFP as a reporter gene in virus expression systems

The success of the GFP protein as a reporter gene is based on several characteristics. GFP is able to generate green fluorescence in living tissue (Chalfie *et al.*, 1994), using UV light as the only 'substrate' required to initiate light emission (An 1985). GFP fluorescence is visibily distinguishable from the red-purple autofluorescence of wild-type plant tissue (An 1985). The GFP protein is non toxic and therefore transformed living tissue expressing GFP may be analysed by spectroscopy, electrophoresis and epifluorescence or confocal microscopy which allows detection at the cell level in real time (Baulcombe *et al.*, 1995). As GFP is also highly resistant to photobleaching it is useful in continuous non-destructive assays (Baulcombe *et al.*, 1995; Tsien 1998), and potenially, during 'in-field' monitoring of agronomically important genes linked to GFP (Harper *et al.*, 1999). There is anecdotal evidence reported by Haselhoff *et al.*, (1997) which indicated that GFP was cytotoxic in Arabidopsis. However this damage may be due to higher than normal levels of hydrogen peroxide that are produced in association with chromophore maturation. Hydrogen peroxide may be produced as a by-product during the period of oxidation that is required before a newly synthesised chromophore matures into its optimum absorbing and fluorescing structure (Tsien 1998). Coding for the GFP protein is derived from the primary amino acid sequence by post-translational modification (Prasher *et al.*, 1992; Cody *et al.*, 1993; Tsien 1998). Mutant GFPs with increased levels

of fluorescence have been produced after targeted amino acid substitutions (Rizzuto *et al.*, 1996; Pang *et al.*, 1996; Niwa *et al.*, 1999). There are two disadvantages associated with this reporter protein. Firstly, observations of GFP fluorescence may be affected by additional background fluorescence originating from nectrotic tissue. Secondly, due to the period of oxidative post-translational modification that is required for GFP maturation (Cody *et al.*, 1993; Heim *et al.*, 1994), GFP fluorescence may be delayed by potentially a day (Baulcombe *et al.*, 1995).

3.1.3 Aim

The primary aim of the following experiment was to investigate the dynamics of TRV spread in the *N. benthamiana* host plant using GFP as a reporter protein. Time course experiments were performed to characterize symptom development in whole plants. Further goals of this experiment were to determine the stability of the GFP gene sequence in the virus genome and to determine whether recovery from infection induced by the recombinant TRV-GFP induced a TRV-GFP-resistance state in the *N. benthamiana* plant. No examples of studies comparing the expression levels of GFP by *Potato Virus X* (PVX) and *Tobacco Mosaic virus* (TMV) and TRV were found in the literature. An experiment was designed to investigate whether the TRV vector was more efficient at expressing GFP in the root system than the alternative expression vectors based on PVX and TMV. This chapter would therefore provide essential background information for subsequent TRV expression studies involving GNA.

3.2 Materials and Methods

3.2.1 The dynamics of GFP expression by TRV-GFP

3.2.1.1 Plant inoculation and symptom identification

The first true leaf of 2 - 4 week old *N. benthamiana* plants was mechanically inoculated according to the method outlined in section 2.2.19. The progress of fluorescence in the leaf induced by TRV-GFP was monitored daily under a long wave UV light source. GFP was identified as zones of bright green, fluorescing leaf tissue as depicted in Figure 3.1. Plant tissue samples were harvested when fluorescent systemic symptoms were recorded. Plant tissue was then ground to slurry with water using a pestle and mortar, and frozen at -20 °C. Infected tissue extract harvested from a transcript inoculated plant was designated as 'first passage' inoculum and used in the following time course experiment as virus inoculum. Infected tissue extract harvested from successive time course experiments were then designated as second, third and fourth passage inoculum. GFP and virus coat protein (CP) were detected in extracts of infected plants by western blotting, by probing with anti-GFP and anti-CP antibodies respectively (2.2.20).

3.2.1.2 First passage of TRV-GFP through the *N. benthamiana* host plant

N. benthamiana plants were coinoculated with TRV/K20/GFP transcripts and total NM RNA (see section 2.2.17 for method of transcript synthesis). The development of fluorescent symptoms was monitored periodically. Only systemic leaf tissue that exhibited highly fluorescent symptoms was selected to be processed for inoculum preparation (first passage inoculum) according to the instructions above.

3.2.1.3 Second passage - an assessment of GFP expression

Eighteen *N. benthamiana* plants were inoculated with leaf extract harvested at 7 dpi from the previous first passage experiment. A leaf showing fluorescent systemic symptoms were harvested, from three plants, each sample date. Leaves were subsequently divided in half. The left half was weighed and homogenized according to the previous section and stored at -20 °C. Total RNA was extracted from the right half according to method 2.2.15, except that the RNA was stored at -20 °C after precipitation in $LiCl_2$.

Total RNA extracts for the 4 and 10 dpi samples were centrifuged at 14 000 *g* for 10 minutes, rinsed in 70 % ethanol, air dried and the resulting RNA pellet re-suspended in 20 µl sterile distilled water. Spectrophometer readings were then used to estimate the total amount of RNA present (2.2.6). The RNA was then diluted to 10 ng/µl and used as a template to perform reverse transcription using oligonucleotide 305 (see 2.1 for nucleotide sequence) (2.2.16). A 50 µl PCR reaction was performed on 10 ng of each cDNA sample: i) 4 dpi; ii) 10 dpi; iii) wild-type TRV/K20 plasmid DNA and iv) TRV/K20/GFPc (1.038 µg/µl plasmid DNA). PCR was performed using oligo 319 (specific for the TRV/K20 RNA2 virus promoter) and 300 (specific for the 3' terminus of TRV/K20 RNA2) to amplify a fragment correlating to the full-length TRV RNA2 molecule (see 2.1 for nucleotide sequence).

A 20 µl volume of the RT-PCR reaction for both 4 dpi and 10 dpi was electrophoresed on a 1.2 % agarose gel. After gel analysis, the 1.4K fragment was sliced out and the DNA extracted according to section 2.2.11. A sequencing reaction was performed as in 2.2.7, and the resulting nucleotide sequence compared with that of GFP.

3.2.1.4 Third passage - TRV stability study

Forty-five *N. benthamiana* plants were inoculated with leaf extract harvested at 4 dpi from the previous second passage experiment. In the previous time course it was found that the third sink leaf above the inoculated leaf predominantly developed the first fluorescent systemic symptoms. Two leaves were subsequently sampled in duplicate, daily, for 23 days until GFP fluorescence had disappeared. Plants in which fluorescence became apparent only on the fourth or fifth sink leaf above the inoculated leaf were excluded from the sample population. For western blots designated '1' and '2', lanes were loaded with 14 µl or 12 µl tissue:laemmli respectively. The experiment was repeated to validate the observations of GFP expression.

3.2.1.5 Fourth passage

Leaf extract from sample dates 4 to 18 dpi, of the previous third passage experiment (3.2.1.4), were each used to mechanically inoculate two *N. benthamiana*

plants. Visible GFP was detected in all leaves used for inoculum. Two plants were mock-inoculated with water. The development of fluorescent symptoms was then monitored daily under UV light, until 23 dpi when the disappearance of GFP indicated that the leaf had recovered from the infection. The recovered leaf was then challenged with the original inoculum. To demonstrate the development of a TRV-GFP infection in a mature leaf an equivalently positioned leaf on a virus-free, mock-inoculated plant was also inoculated with infective sap (4 dpi). The inoculated leaves were then monitored daily for the appearence of GFP (fluorescent lesions).

3.2.1.6 The dynamics of GFP expression by TRV compared *to Tobacco Mosaic Virus* and *Potato Virus X virus* expression vectors

The following experiments were designed to compare the dynamics of GFP expression in roots and leaves inoculated with TRV-GFP relative to two other commonly used virus vectors: *Tobacco Mosaic Virus* (TMV) and *Potato Virus X* (PVX).

N. benthamiana plants were inoculated with first passage inoculum originating from either TRV-GFPc, TMV-GFP or PVX-GFP transcript-inoculated plants. Inocula for PVX-GFP and TMV-GFP were supplied by Dr Stuart MacFarlane. The progress of fluorescent mosaic leaf symptoms induced by each virus was monitored daily under a long wave UV light source. Total root mass and systemic leaves (half of each fluorescent leaf) were then sampled in duplicate, at daily intervals, 1 day after GFP lesions were evident on the inoculated leaf. For TMV-GFP infections, the inoculated leaf was sampled at 4 and 5 dpi. Tissue extracts were prepared as described in 3.2.1.1.

The time course comparing TRV-GFP and PVX-GFP was repeated, as the progress of the former infection was noticeably more rapid than the latter. To account for the differences in symptom development TRV-GFP inoculations were carried out 5 days after the PVX-GFP inoculations. The delayed inoculation allowed the leaf and root fluorescence, in both infections, to be compared at optimum systemic leaf fluorescence. Systemically infected leaf and root tissue was harvested at 6 dpi and 9 dpi for TRV-GFP and PVX-GFP respectively and processed as above.

3.3 Results

3.3.1 Time course observations for *Tobacco rattle virus* infection

The time course of a TRV infection was recorded for three serial passages of TRV-GFP. The most informative data was obtained for the third passage time course (the stability study), which sampled 45 plants at 2 day intervals for a period of 23 days. Whole plants were screened by viewing plants under UV, and the pattern of GFP expression of the study leaf (the third sink leaf above the inoculated leaf) was investigated by tissue sampling. Under UV light, discrete green fluorescent lesions were first detected on the inoculated leaf at 2 dpi, for all 45 plants. A systemic fluorescent lesion was detected on the study leaf of 2 plants at 4 dpi indicating that systemic virus spread had occurred. The above observations were also supported by those made in the first and second passage time course studies, although for the former experiment systemic lesions were also detected after 3 dpi. At 5 dpi, intense green fluorescence was uniformly visible across the whole lamina surface of the study leaf. This pattern of infection was displayed by 35 of 45 infected plants. A photograph of a systemically infected *N. benthamiana* plant is shown in Figure 3.1. There was a noticeable decrease in the intensity of GFP fluorescence in the study leaf between 7 dpi and 8 dpi. At 9 dpi, GFP fluorescence had faded in the study leaf of 9 plants, but for a further 11 plants the fluorescence had deteriorated to approximately 10 % of original value (estimated by eye). Fluorescence of the leaf lamina then gradually disappeared in this experiment until 14 dpi, after which point only the trichomes appeared intensely fluorescent. Two fluorescent trichomes were evident at 18 dpi.

Figure 3.2 presents the western blots for the third passage time course experiment and indicates that detection of CP (Blot A) and GFP (Blot B) both correlate well with visual assessment of fluorescent symptoms. CP and GFP were both initially detected at 4 dpi. Similar levels of GFP were detected in the study leaf between 4 dpi and 13 dpi, although western blotting did not show the change in GFP intensity that was observed under UV light over this period. However, a faint and highly variable GFP signal was detected between 14 dpi and 19 dpi, which correlates with the gradual disappearance of GFP in the leaf lamina and the appearance of highly fluorescent trichomes. In contrast, CP

24 mm

Figure 3.1 *N. benthamiana* systemically infected with TRV-GFP at 7 dpi, illuminated under UV light. Arrow identifies the study leaf which shows a uniform distribution of GFP across the whole leaf lamina. Photograph also demonstrates the uniform pattern of GFP expression in roots.

was detected in leaf extracts until 22 dpi, which shows that the GFP protein degrades more rapidly than CP.

An identical pattern of GFP expression to that described above was observed when the experiment was repeated, although here a single, highly fluorescent trichome was detected on a previously fluorescent study leaf at 23 dpi.

The presence of GFP fluorescence/symptoms in the first passage TRV infection indicated that the TRV/K20/GFPb transcripts were infectious when inoculated on to *N. benthamiana* plants. Fluorescent leaf tissue was harvested for inoculum at 7 dpi consequently limiting the time course. However, only 4 of the 6 inoculated plants were infected. This may be related to the quantity of RNA2 transcript used for inoculation, as all sap-inoculated plants in the subsequent experiments became infected.

Systemic GFP fluorescence was observed until 10 dpi in the second passage time course experiment. The removal three leaves per sample date also limited the period of the time course. All visual observations of GFP were supported by western analysis of infected tissue extracts. The resulting blot is not presented, but was similar to that shown

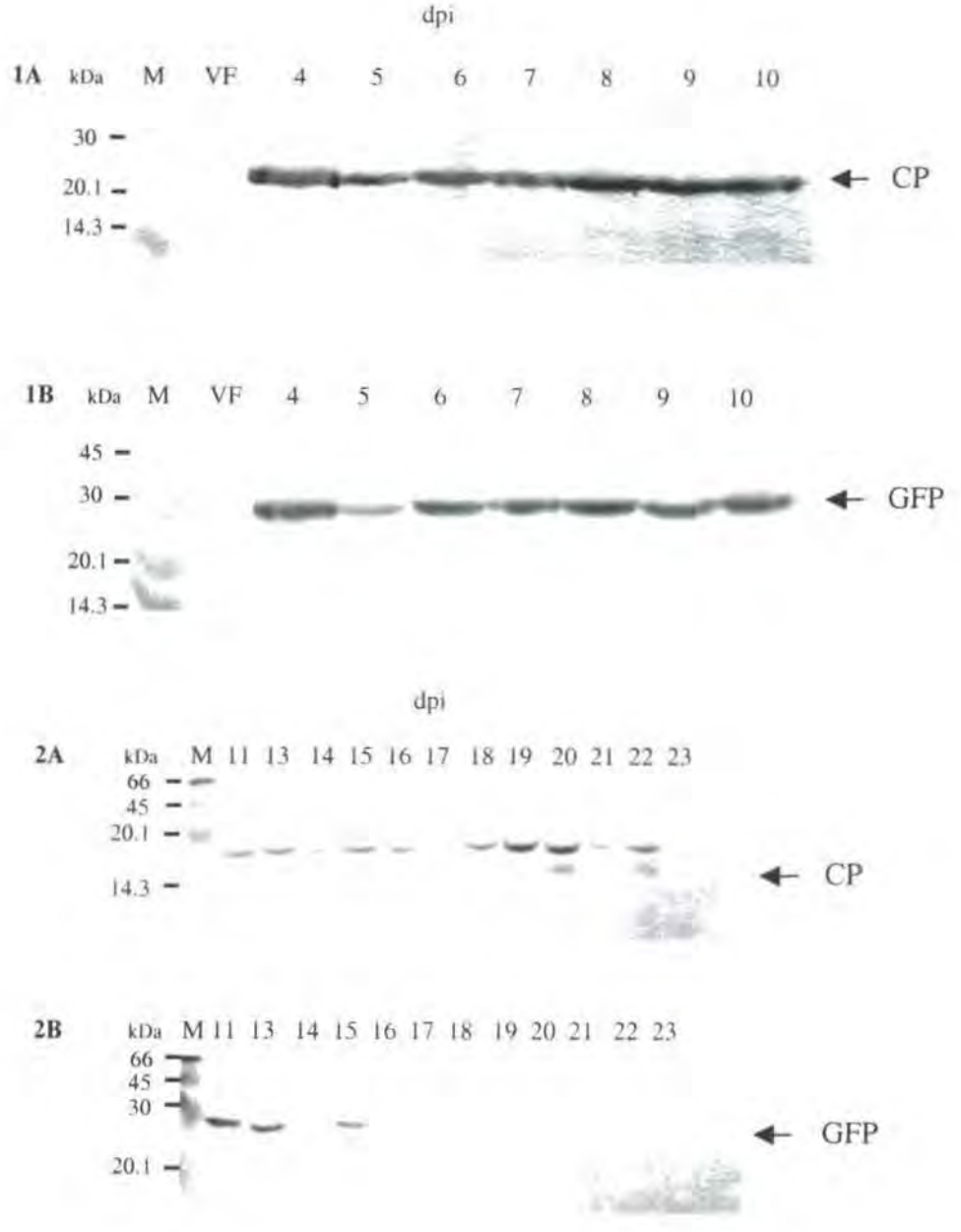


Figure 3.2 Western blot of time course of GFP expression by TRV-GFP in *N. benthamiana* leaves. M denotes size marker and VF denotes a virus-free leaf sample. Lanes are labelled in days postinoculation. Panels 1A and 1B are leaf samples harvested between 4 dpi and 10 dpi and panels 2A and 2B are samples harvested between 11 dpi and 23 dpi. Blots 1A and 2A were probed with TRV coat protein specific antibodies. Blots 1B and 2B were probed with GFP-specific antibodies.

in Figure 3.2 except that a positive GFP signal was detected between 4 dpi until 10 dpi. Tissue sampling in this experiment did not extend to cover observations of GFP degradation. A summary of the western blot data for the third time course (third passage) is presented in Figure 3.7.

3.3.3 Pathogen derived resistance is induced by TRV-GFP (fourth passage)

Inoculum used for this study originated from leaves between 4 and 18 dpi of a time course. The blot shown in Figure 3.2 shows the variation of CP and GFP detected in the inoculum at these dates. Systemic leaf fluorescence was observed after inoculation with the sap treatment. At 23 dpi, systemic leaf GFP had disappeared and the recovered leaves were challenged with original inoculum. GFP lesions were not detected on mock-inoculated leaves (Figure 3.3 i), or on the recovered leaves (Figure 3.3 iii). Fluorescent lesions like those shown in Figure 3.3 ii) were only detected on the inoculated leaf of a virus-free plant, under UV light, at 3 dpi. The former observation indicated that the inoculum was infective.

Figure 3.3 Detection of local GFP fluorescent lesions, under UV light, on *N. benthamiana* leaf infected by third passage TRV-GFP inoculum after 3 days (ii). Fluorescence was not detected on a mock-inoculated leaf (i), or, after challenging a recovered systemically infected leaf with TRV-GFP inoculum (iii).

3.3.4 Detection of gfp by RT-PCR in leaf extract harvested from a second passage TRV-GFP infection

Leaf samples collected on 4 and 10 dpi of the second-passage time course were found to contain a total of 127 µg and 150.48 µg total RNA respectively. Total RNA was then used as template to perform RT-PCR for each sample date. Five µl of reaction product was then analyzed on a 1 % agarose mini gel at 140 V and visualized using an UV transilluminator, and the results shown in Figure 3.4. The expected products were generated following amplification of wild-type PpK20 TRV/RNA2 (2 K fragment in lane a) and TRV-GFPc (1.2 K fragment in lane a) plasmid DNA. However, for both the second passage 4 dpi and 10 dpi TRV-GFP sap samples (lane c and lane d respectively), an expected fragment of 1.2 K and an additional larger fragment of approximately 1.4 K was also generated. Nucleotide sequence analysis indicated that a full-length GFP sequence was contained in the large fragment.

Figure 3.4 Detection of TRV-GFP RNA2 by RT-PCR. Lane 1 and 6, DNA ladder. Fragments of viral RNA2 amplified from plasmid DNA: lane 2, wild-type PpK20 TRV RNA2 and lane 3, TRV-GFPc. Viral RNA2 amplified from total RNA isolated from infected leaves after 4 dpi (lane 4) and 10 dpi (lane 5)

3.3.5 A comparison of GFP expression in roots and leaves by TRV, TMV and PVX virus expression vectors

The differences in time course of systemic infection between TRV and TMV or PVX virus vectors were determined by comparing GFP expression in leaf and root tissue samples. A summary of the western blot data for these studies are presented in Figure 3.8.

3.3.5.1 Greater amounts of systemic GFP are expressed by TRV-GFP than TMV-GFP in the initial stages of infection

In this time course experiment the progression of the initial virus infection was more rapid in *N. benthamiana* plants inoculated with TRV-GFP compared to TMV-GFP. Discrete lesions were present on the TMV-GFP inoculated leaf at 4 dpi, whereas systemic leaf symptoms were detected in TRV-GFP infections at this time. However, systemic GFP expressed in the study leaves by TRV-GFP had peaked in all 6 plants by 6 and 7 dpi, and fading was apparent at 8 dpi. In contrast, systemic leaf fluorescence expressed by TMV-GFP was detected in only 1 of 24 plants at 6 dpi and only half of the plants after 8 dpi, although the infection continued to develop after 14 dpi (the last observation). The blot (Figure 3.5 A) indicated that the amount of GFP detected in leaves systemically infected with TRV-GFP is greater at both 4 and 8 dpi than at 6 dpi for plants infected by TMV-GFP. This difference in GFP expression may be due to the differences in symptomology. For example, TMV-GFP spread across the leaf in a speckled or mosaic pattern that followed the vein architecture of the leaf (this difference may also account for the variability between samples), whereas, TRV-GFP spread uniformly across the leaf lamina in a solid block of fluorescence and was present in the veins and lamina of the study leaf. The intense brightness of GFP observed in TRV-GFP infected leaves may also be due to the solid area of fluorescing GFP.

GFP expression was observed in the roots of TRV-GFP infected plants between 4 and 8 dpi compared to 8 dpi for TMV-GFP infections. The observation was supported by the root blot presented in Figure 3.5 B, which also shows that the level of GFP expressed by TRV-GFP at 4 and 8 dpi was greater than that expressed in TMV-GFP infected root

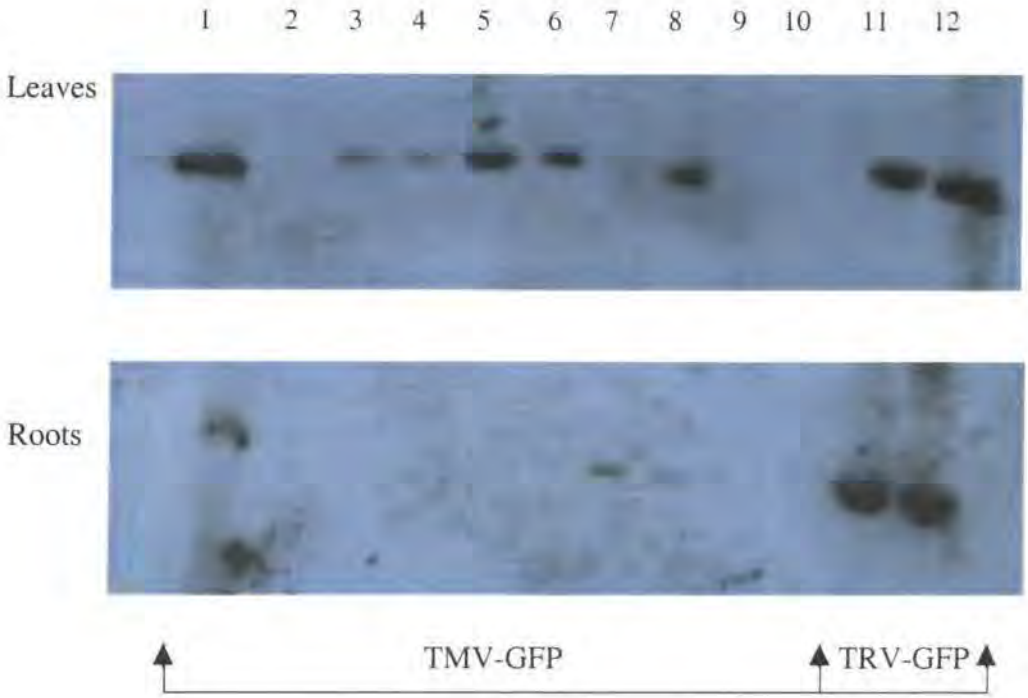


Figure 3.5 Western blot to illustrate differences in GFP expression in *N. benthamiana* by TMV-GFP compared to TRV-GFP. Top panel is leaf extracts. Lower panel is extracts of whole root system excluding hypercotyl. Lane 1, positive control (TRV-GFP at 7 dpi). Lanes 2, uninfected tissue extract. Lanes 3 and 4, TMV-GFP inoculated leaves harvested at 4 dpi and 5 dpi. Lanes 5-8, TMV-GFP infected plants showing systemic fluorescence between 6-9 dpi. Lanes 9-10, spacer lanes. Lanes 11-12, tissue extracts from highly fluorescent study leaves systemically infected with TRV-GFP at 4 dpi and 8 dpi respectively. Lanes were loaded with 12 µl tissue:laemli sample buffer. Blots were probed with anti-GFP antibodies.

extract. The blot also suggests that GFP expression, in terms of yield in whole plants infected by TRV-GFP, was greatest in the root tissue. GFP expression in the roots by TRV-GFP was also spread more evenly through the whole root mass. In contrast, GFP expressed by TMV-GFP was often patchy and concentrated to a small number of roots.

3.3.5.2 TRV-GFP expresses greater amounts of GFP in root tissue than PVX-GFP

The first time course experiment for TRV-GFP and PVX-GFP indicated that the progress of a systemic infection by the former virus, in *N. benthamiana* plants, was more rapid than PVX-GFP despite fluorescent lesions being detected on the inoculated leaf at 4 dpi for both virus vectors. Systemic fluorescent symptoms were detected under UV light at 6 dpi for TRV-GFP (which is comparable to previous studies) compared to

9 dpi for PVX-GFP. The time course observations for PVX-GFP were similar to those recorded by Roberts (1999). To ensure that the systemic symptoms in both infections were compared at optimum leaf fluorescence, the TRV-GFP inoculation was delayed by 4 days. Figure 3.6 demonstrates the pattern of GFP expression in whole plants, when viewed under UV light at optimum leaf fluorescence, after inoculation by TRV-GFP (6 dpi, A) and PVX-GFP (9 dpi B). Regions of reddish-purple represent tissue autofluorescence and show areas that were not infected by the virus. GFP fluorescence in the PVX-GFP inoculated plant was less intense than GFP expressed by TRV-GFP. The roots of the TRV-GFP inoculated plant displayed an intense, solid region of GFP fluorescence that extended from the crown of the stem to the root tips, showing that the roots were heavily infected with the virus. In contrast, GFP fluorescence in the roots of PVX-GFP inoculated plants was absent or very feint. Differences in GFP expression in leaf and root extracts of six individual plants showing highly fluorescent, systemic leaf symptoms were apparent after analysis by western blotting. The total root mass and half of each fluorescent systemic leaf were harvested from six *N. benthamiana* plants that showed highly fluorescent leaf symptoms. The western blots presented in Figure 3.7 illustrate that similar levels of GFP were expressed in the leaves of plants infected by TRV-GFP and PVX-GFP (blot A). However, the level of GFP expressed in TRV-GFP infected root tissue, in blot B, is 10- to 25- fold higher than in roots infected by PVX-GFP when compared by densitrometric analysis.

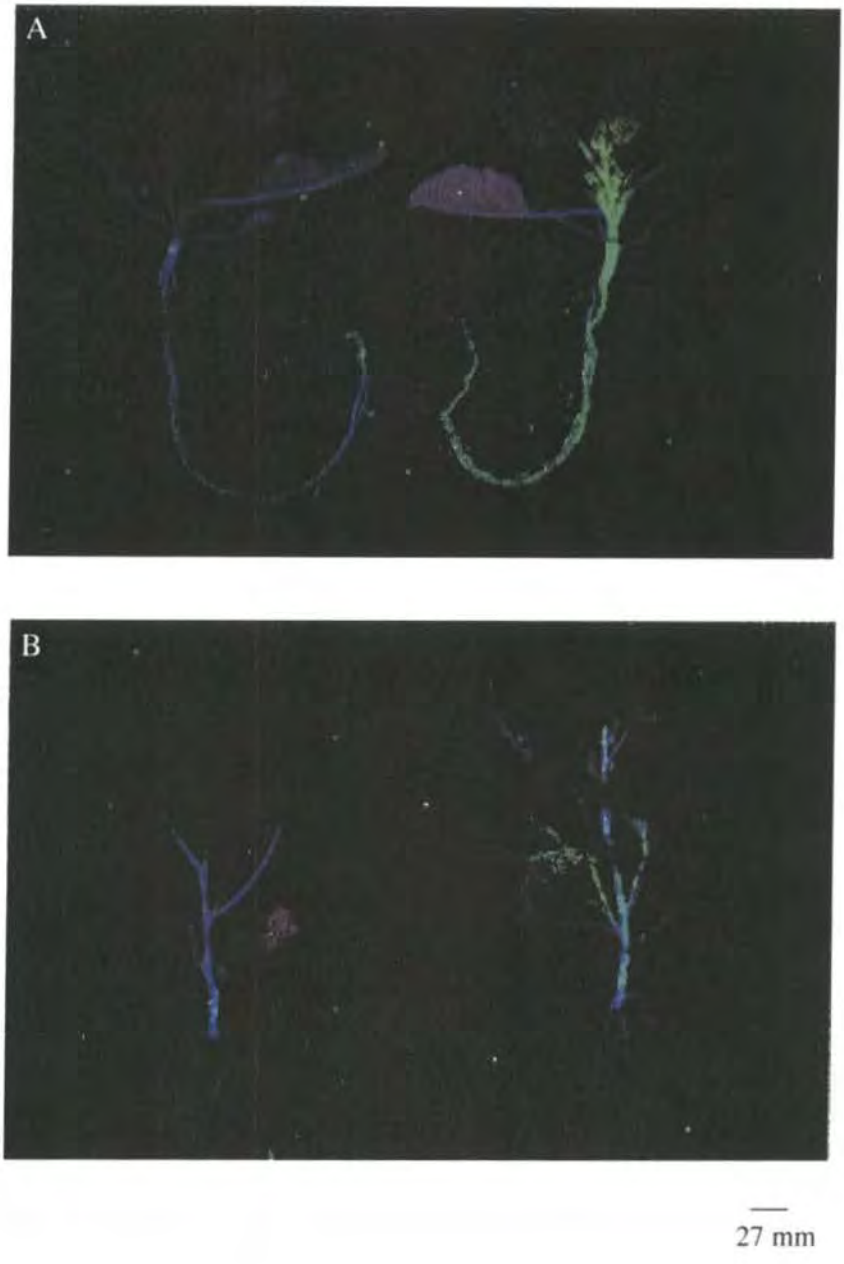


Figure 3.6 Visualization of the pattern of GFP expression in whole *N. benthamiana* plants when illuminated under UV light. Mock-inoculated plant (left) compared with A) TRV-GFP at 6 dpi (right) and B) PVX-GFP at 9 dpi (right). Red-purple colouration is autofluorescence of uninfected tissue.

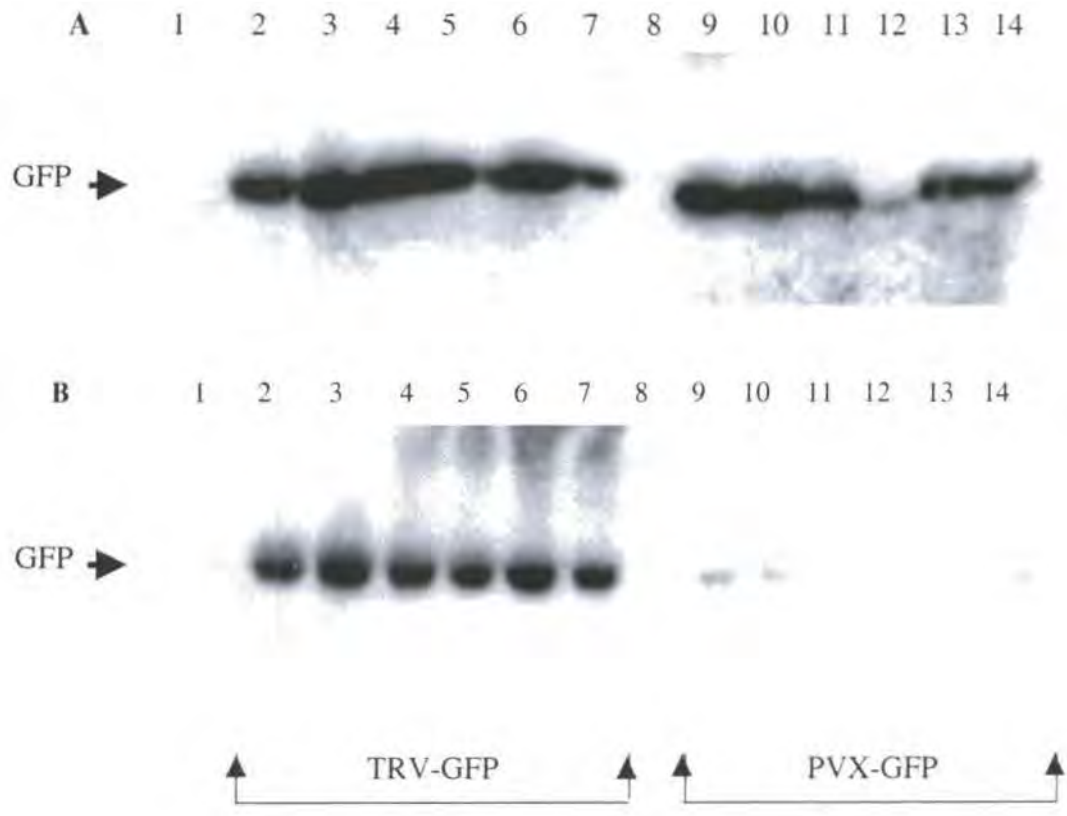
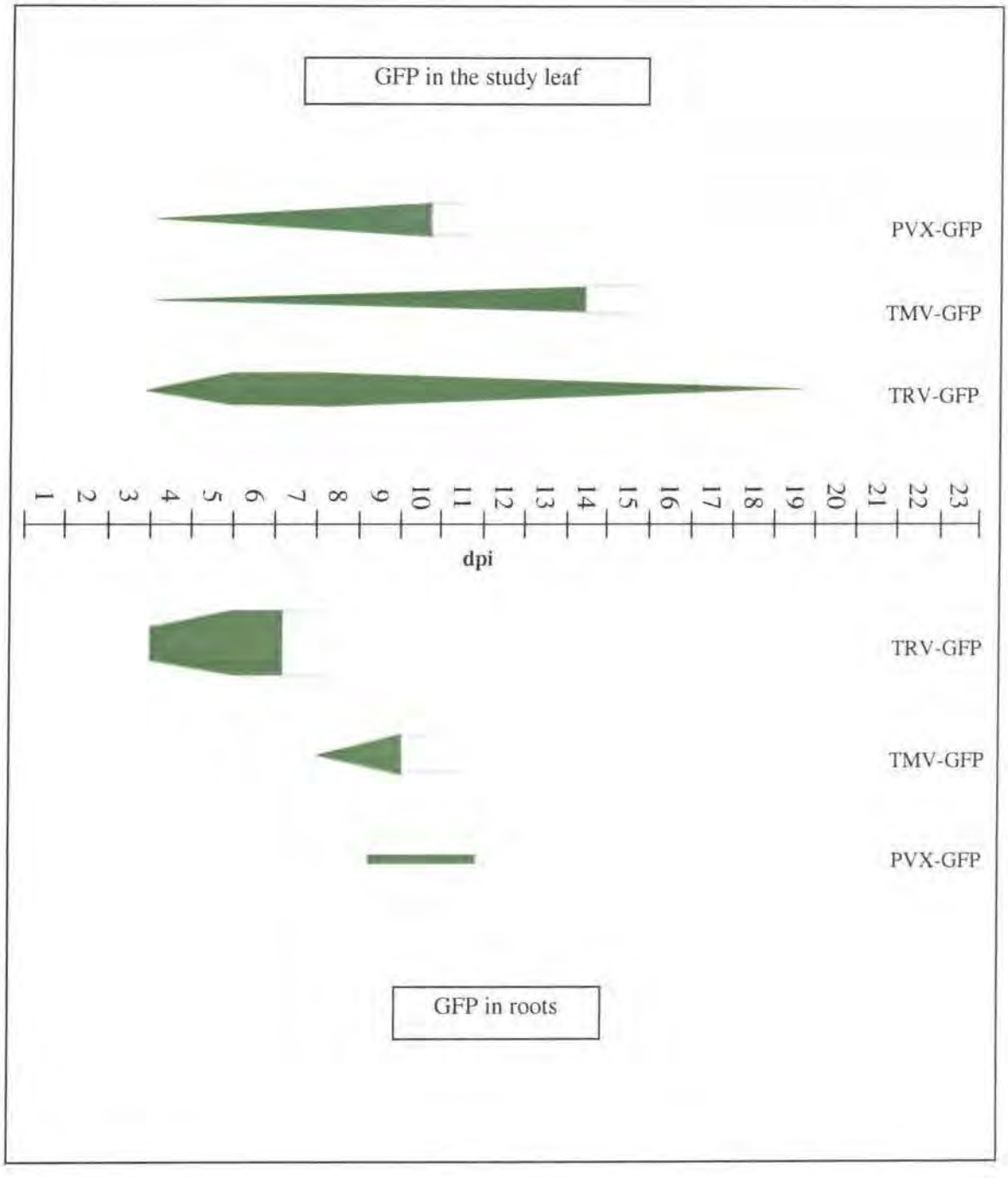


Figure 3.7 Western blots comparing GFP expression in six individual *N. benthamiana* plants infected with TRV-GFP or PVX-GFP. Blot A is extracts of leaves showing highly fluorescent systemic GFP symptoms. Blot B is extracts of the whole root system excluding the hypercotyl. Lanes 1 and 8, uninfected tissue samples. Lanes 2-7, TRV-GFP infected plants at 6 dpi. Lanes 9-14, PVX-GFP infected plants at 9 dpi.

Figure 3.8 Summary of systemic GFP fluorescence in leaves and roots recorded during time-course experiments for TRV-GFP, TMV-GFP and PVX-GFP infections in *N. benthamiana*



3.4 Discussion

3.4.1 Analysis of GFP expression by the TRV expression vector

The jellyfish GFP protein has previously been used as a virus expression reporter protein to analyze the dynamics of virus spread in a host plant. In the following time course experiments the GFP marker protein was also used to demonstrate the progress and dynamics of TRV movement in *N. benthamiana*. This data was required as background information for the subsequent bioassay experiment, but could ultimately be used as a reference for the expression of any foreign protein by the TRV expression vector.

The third TRV-GFP time course produced the most detailed description of GFP and CP expression in the study leaves as tissue was sampled until the GFP fluorescence had disappeared. In this experiment a greater number of plants were infected after inoculation with second passage inoculum compared with plants infected with first passage inoculum. This difference possibly reflects the reduced infectivity of inoculum comprised of transcript RNA rather than virus particles that are present in infected leaf extracts. Typically local fluorescent lesions were produced on the inoculated leaf after 2 dpi, with the first systemic symptoms being observed on a sink leaf (the study leaf) at 4 dpi. Optimum GFP fluorescence was observed between 5 dpi and 7 dpi, leaf fluorescence faded from 8 dpi to 14 dpi and only some trichomes were fluorescent up to 18 dpi. Blots of leaf samples harvested from this time course suggested that there was a good correlation between the visual observations of GFP made under UV light and western analyses using antiserum against virally expressed proteins (GFP and CP). This observation suggests that immunological detection methods do provide an accurate account of virus infection which would be useful as symptom development in TRV/K20 infected *N. benthamiana* plants is mild and difficult to detect by eye.

In the case of both GFP and CP, the decline of the signal is dependent on the nature of the protein, the phase of active virus replication in the infected leaf and possibly the developmental stage of the leaf. The signal for virus coat protein was consistently detected in sap extracts analyzed by western blotting between 4 dpi until 22 dpi, suggesting that the viral particles were more stable in plant sap than GFP. However, it also suggested that the

leaf had fully recovered from the TRV-GFP infection at this point. The disappearance of GFP is probably due to its high solubility in the absence of incusion bodies (Tsien 1998).

A further aim of this research was to determine the stability of a non-viral sequence, inserted into the virus genome, after serial passages through a host plant. The results from these experiments show that GFP fluorescence was detected visually, and by western analyses, after four serial passages of the TRV-GFP expression vector in *N. benthaminana* (the final passage). This suggested that the GFP open reading frame (ORF) was maintained stably in the virus genome despite the detection of a larger RNA2 species.

Plants infected with one strain of virus may protect the host plant from infection from an identical or related virus (Harrison 1958). Challenging a recovered, previously systemically infected leaf from a TRV-GFP infected plant (fourth passage) with identical inoculum did not induce fluorescent lesions on the inoculated leaf. This indicated that the plants were not susceptible to a secondary TRV-GFP infection, this suggested that cross-protection had occurred since protection against re-infection was complete (Fulton 1986; Lindbo *et al.,* 1993).

3.4.1 Insertion of GFP in the TRV expression vector was stable

Although virus observations suggest that GFP was stably integrated into the TRV genome. Analysis of the RT-PCR products for the 4 and 10 dpi sample dates indicated that a second TRV-GFP RNA2 molecule had accumulated during the second passage of the virus vector. This fragment was approximately 200 bp larger than the expected product (1.2 K), although sequence analysis confirmed that a full-length GFP sequence was present in the larger RNA2 fragment. Given that the first passage TRV-GFP inoculum was induced from a pure, uncontaminated source RNA2 (TRV-GFP RNA2 run-off transcripts originating from a cDNA clone consisting of the entire viral RNA2 sequence down stream of a T7 promoter). This suggests that the second RNA2 species might be the result of a recombination event, *in planta,* during the life cycle of the virus.

RNA recombination is a phenomenon that is commonly observed in positive sense RNA viruses (Lai 1992). However, the exact molecular mechanism of this process is

unclear (Figlerowicz 2000). It is thought that a 'copy choice mechanism' is involved in RNA recombination during the synthesis of nascent RNA (negative sense) molecules, including DI RNAs (Lai 1992). Secondary structures, which may occur in either the template or the nascent molecule, have previously been associated with transcriptional pausing, and may result in the production of incomplete transcripts. Close proximity to a different RNA molecule may allow recombination to occur and for transcription to continue. This may be due to either a physical linking of the two RNA templates, or possibly to the formation of a heteroduplex caused by complementarity between the secondary structures, causing the switching of RNA polymerase, or RNA polymerase and transcription machinery, to the new template. At the 'cross over' site, the recognition of the transcription re-initiation site is probably again determined by secondary structures rather than a specific sequence, although the presence of homologous sequences may aid the RNA polymerase to re-attach. Template switching may occur at some distance from the actual 'cross over' site. In the case of DI RNAs, it was thought that nonhomologous recombination, between two different RNA molecules, was the result of polymerase 'jumping', at secondary structures during transcription (Lai 1992).

A study by Hernández *et al.,* (1996) using RT-PCR, revealed that deletions of structural and nonstructural genes in TRV PpK20 RNA2 commonly appear after a single passage through tobacco plants. The resulting smaller RNA2 species, known as DI (defective interfering) RNAs, are derived from the original RNA2 genome and accumulate over serial passages becoming dominant in further passages thus outcompeting the genomic RNA2 species, without affecting the RNA1 species. No small DI RNA2 species were detected by RT-PCR in twice passaged sap during this current TRV-GFP experiment. However, a larger 1.4 K fragment was detected which suggests that the larger RNA2 species may not be a 'typical' TRV DI RNA. Although larger DI RNAs have been demonstrated to accumulate in protoplasts infected by *Tombusviruses*, these rapidly transform to smaller DI molecules (White and Morris, 1994). Hernández *et al.,* (1996) found that the genomic regions targeted by recombination events in the TRV PpK20 isolate were the CP gene, the 40 K* gene and the 32.8 K gene (nonstructural genes 2b and 2c respectively) (Hernandez *et al.,* 1996; Visser *et al.,* 1999*). It was also noted that these

genes were flanked at the borders by short nucleotide repeates resembling the 5' region of the genomic and subgenomic TRV RNAs. Also there were regions surrounding the deletion sites that could potentially fold into secondary stem loop structures, although data for the latter hypothesis was not published (Hernandez *et al.*, 1996; Visser *et al.*, 1999*). This observation raises the interesting possibility that replacement of the non-structural genes in the virus expression vector has reduced the number of possible 'cross over' sites or 'hot spots' that may form secondary structures required for recombination.

As a primary aim of this research was to show stability of the GFP sequence in TRV RNA2, the effect of serial passages on the formation of additional RNA species was not fully investigated in this research. Given that recombination is more likely to occur between closely related viral sequences (Lai 1992), but that the template and nascent RNA2 molecules both contain the GFP sequence which are potential sites for homologous recombination. Further studies based on sequencing of DI RNAs produced by TRV-GFP infections would be be required to determine the origin of the extra sequence inserted into the 1.4 K RNA2 fragment. A comparison of the sequence of different DI RNAs might also identify recombination 'hot spots' that could also be targeted for deletion or mutation during the design of virus expression vectors. These future experiments could be used to establish whether the stability of the GFP sequence in the RNA2 molecule was due to the replacement of the recombinant-sensitive non-structural genes, or, to the virus vector expressing distantly related genes rather than virus genes.

Expression of the marker protein GFP provided a tool to compare the rates of TRV movement, and therefore systemic infection, of two other established virus expression vectors based on TMV and PVX. Firstly, time course experiments indicated that temporal differences in GFP expression exist between the three viruses. In the case of TMV-GFP, the results presented in Figure 3.5 indicated that although fluorescent lesions had appeared on the inoculated leaf of both infections after 4 dpi, systemic infection was more rapid in plants infected by TRV-GFP (100% of plants systemic at 6 dpi) compared to only 50 % of the TMV-GFP infected plants at 8 dpi. Observations made under UV light were supported by western analyses. In the case of PVX-GFP, inoculation of plants by TRV-GFP required a delay of 4 days to allow a comparison of leaf fluorescence in

systemic infections. The PVX-GFP time course observed in this study was similar to that recorded by Roberts (1999).

Secondly, western analyses indicated a variation in the potential for GFP expression in both leaves and roots by the different virus vectors. Blots of sap extracted from the systemically infected study leaf indicated that, although similar levels of GFP were expressed by each of the viruses in this tissue, there were differences in the pattern of GFP expression across the leaf lamina. For example, in both TMV-GFP and PVX-GFP infections the fluorescence was observed as a mosaic compared to uniform blocks of GFP expressed by TRV-GFP. Blots of root extract also indicated that the potential for GFP expression in root tissue was greatest for TRV-GFP when compared to both TMV-GFP and PVX-GFP (Figures 3.5 and 3.7 respectively). These differences may again be related to the different patterns of GFP expression in the root system, but as each GFP molecule only produces one fluophore (Tsien 1998), the differences in the observed intensity of GFP fluorescence expressed in the roots by TRV-GFP and PVX-GFP are also quantitative. For example, highly fluorescent, uniform GFP expression in roots was produced during a TRV-GFP infection, whereas only low levels of GFP were expressed by PVX-GFP, and GFP expression was patchy or concentrated to a small number of roots in TMV-GFP infections.

3.4.3 Conclusion

Collectively, these results demonstrate that the TRV expression vector has several advantages over the other tested virus expression vectors for application as the delivery agent of pesiticidal proteins.

1) The TRV expression vector induces a more uniform systemic infection in *N. benthamiana* compared to TMV or PVX. This would be an important factor for pathogen bioassays as it would minimize the temporal and positional differences in virally-expressed proteins and therefore promote a more standardized test. Mosaic or patchy symptoms, like those induced by TMV-GFP and PVX-GFP, would provide feeding pathogens with a choice of uninfected or infected tissue. Solid areas of GFP fluorescence, like those induced in the study leaf by TRV-GFP, would therefore limit the variation in

exposure of the feeding pathogen to virally-expressed proteins. The 'study leaf' was also repeatedly infected by the virus vector which suggests that this leaf could be used as a test arena for leaf feeding pathogens.

2) A TRV-GFP systemic infection was more rapid compared to TMV (by minimum of 2 days) or PVX (by 4 days). Thus allowing the bioassay to be initiated quickly at an earlier phase of plant developemnt

3) TRV-GFP expressed GFP more efficiently in root tissue and at higher levels than either TMV or PVX confirming the potential of TRV-GFP as a virus expression vector with root expression capabilities. This indicated that like above, the root feeding pathogens would be directly exposed to the virally-expressed proteins.

4) The wide host range of TRV (section 1.3.2.2) suggests that this virus expression vector could be developed to allow gene expression in several host plant systems. For example, systemic GFP has been detected in tomato, sugar beet and *Arabidopsis* infected by the TRV expression vector and in tomato for the PepRSV expression vector (MacFarlane and Popovich 2000).

Chapter 4

The expression of snowdrop lectin from Tobravirus (*Tobacco Rattle Virus)* expression vectors

4.1 Introduction

In planta, the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) has been isolated from bulb, vegetative and flower tissue (van Damme *et al.,* 1990a, 1991ab, 1992; van Damme and Peumans, 1990a, 1996) where its physiological role has been associated with storage (van Damme *et al.,* 1991ab; van Damme and Peumans, 1996), ovary development and passive constitutive defence (van Damme and Peumans, 1990a; van Damme *et al.,* 1992). The mulitfunctional role of lectins like GNA has been attributed to several factors including its presence in tissues as a complex mixture of isolectins (LEC 1, 2, 3, 5 and 8), developmental regulation (van Damme and Peumans, 1990b; van Damme, *et al.,* 1991b) and unique carbohydrate-binding properites and specificities. GNA is a 50 kDa homo-tetramer (van Damme, *et al.,* 1987, 1991ab) that recognizes twelve mannose residues at highly conserved binding sites on its surface (Hester, *et al.,* 1995; Hester and Wright, 1996; Barre *et al.,* 1996). The GNA molecule therefore has the capabilities to function as a recognition molecule and decipher the monosaccharide and oligosaccharide sugar code that provides the code for inter- and intracellular recognition processes (Gabius 2000).

In terms of research, a major interest in lectins was derived from their ability to discriminate between monosaccharides and oligosaccharidesis, plant and non-plant and therefore their defence-related properties (see section 1.4.2.3). Discovery of genes with pesticidal properties (or other beneficial genes) could therefore be introduced into crop species using recombinant DNA techniques and provide 'novel' protection from plant pathogens where natural resistance was limited (Goldstein and Porestz 1986; de Ponti and Mollema, 1992; Gatehouse, *et al.,* 1993; Horsch, 1993). For analysis of gene function *in planta*, foreign genes are typically assessed in bioassays that are performed on transformed plants generated from Agrobacterium transformation. Evaluation of potential pesticidal genes using a virus-based expression system would therefore provide an intermediate step in the appraisal of genes with defence-related properties.

4.1.2 Aim

The aim of the following series of experiments was to investigate the characteristics of GNA expression by the TRV virus vector and to demonstrate that in this system, the GNA monomer was able to assemble into its characteristic tetrameric form. The pattern of GNA accumulation in *N. benthamiana* leaf and roots was to be investigated using time course experiments and tissue squashes. These experiments were then extended to determine the effect of plant size and higher temperature. The purpose of two TRV mutant expression vectors (expressing a truncated GNA protein and TRV-ΔGFP) was to serve as treatments for future bioassay experiments. Further objectives of this study were to investigate their expression and to confirm biological activity. The overall goal of this chapter was to provide further data on foreign protein expression by the TRV vector that would support the application of this virus expression system in insect or nematode bioassays.

4.2 Materials and Methods

4.2.1. Detection of foreign proteins expressed by the TRV expression vector in *N. benthamiana* plants

The first true leaf of 2 - 4 week old *N. benthamiana* plants (grown in compost) was coinoculated with 1) TRV/K20/GNA, 2) TRV/K20/fsGNA or, 3) TRV/ΔGFP transcripts and total NM RNA according to the method outlined in section 2.2.19 (see below). Synthesis of transcript RNA is described in section 2.2.17. Inoculated plants were grown in glasshouse conditions. The study leaf (third sink leaf above the inoculated leaf) was collected according to the pattern of fluorescent systemic symptoms recorded in Chapter 3 (section 3.3.1). The study leaf and roots were collected and ground to slurry with water using a pestle and mortar, and frozen at -20 °C. Sap extracts were then analyzed by western blotting. Lanes were loaded with 14 µl or 12 µl tissue:laemmli buffer. GNA and CP proteins were detected in extracts of infected plants by western blotting, by probing with anti-GNA and anti-CP antibodies respectively (2.2.20).

1) Half of the study leaf (right) and roots from six TRV/K20/GNA transcript inoculated plants were collected at 10 dpi and processed as above. Fourteen µl tissue:laemmli buffer were analyzed by western blotting. Six *N. benthamiana* plants were then mechanically inoculated with TRV-GNA infected root extract (first passage sap) collected from the previous experiment. Leaf and root tissue was collected at 7 dpi and 14 µl tissue:laemmli buffer was analyzed by western blotting.

2) Root tissue from six TRV/K20/fsGNA transcript inoculated plants was collected at 9 dpi and compared to root extract collected from TRV/K20/GNA inoculated plants at 7 dpi. Twelve µl tissue:laemmli buffer was analyzed by western blotting. A BLAST database search at w.w.w.nptl.gov was carried out for the frame-shift GNA nucleotide sequence between position 249 and 570.

3) Root tissue from four TRV/ΔGFP transcript inoculated plants was collected at 7 dpi. Twelve µl tissue:laemmli buffer was analyzed by western blotting. Infected root extract (first passage) collected from each of the infected plants was then used to mechanically inoculate two *N. benthamiana* plants. Root tissue was collected at 7 dpi and 12 µl tissue:laemmli buffer analyzed by western blotting.

4.2.2 Detection of tetrameric GNA in tissue extracts

N. benthamiana plants were inoculated with TRV/K20/GNA transcript according to the method described in 4.2.1. Two plants were mock-inoculated with water. Root and leaf tissue was collected at 9 dpi. IEF columns were loaded with 20 µl leaf extract or 14 µl of root extract and analyzed by non-denaturing two dimensional isoeletric focusing (method is described in section 2.2.23). Blots were probed with anti-GNA antibodies.

4.2.3 The dynamics of GNA expression by TRV-GNA

The following time course experiments were designed to determine the dynamics of GNA expression in roots and leaves infected with TRV-GNA. *N. benthamiana* plants were inoculated with first passage inoculum unless stated otherwise. Total root mass and systemic leaves were then sampled in duplicate, at daily intervals. Tissue extracts were prepared and 12 µl analyzed by western blotting as described in 4.2.1.

4.2.3.1 Assessment of GNA expression in *N. benthamiana* roots and leaves

Sixteen *N. benthamiana* plants were inoculated with first passage root extract collected at 9 dpi. The root system (not including the hypocotyl) and two study leaves were then collected at three day intervals for 24 days.

4.2.3.2 GNA expression in roots of *N. benthamiana* plants propagated in sand

N. benthamiana plants were germinated in compost and transferred into 30 ml pots containing sieved sand for further growth (see the materials and methods for Chapter 5). After 27 days of growth, forty-four plants were mechanically inoculated with TRV-GNA infected tissue extract (second passage). Two root systems were collected at daily intervals between 2 dpi and 23 dpi.

4.2.3.3 GNA expression in roots of *N. benthamiana* at a tropical temperature

The above experiment was repeated except that at 8 dpi all plants were transferred to a temperature controlled glass-house set at 26-28 °C. Root tissue was collected at daily intervals between 3 dpi and 18 dpi.

4.2.3.4 Tissue print of TRV-GNA, fsGNA and TRV-ΔGFP infected roots

N. benthamiana plants were inoculated with TRV-GNA, TRV-fsGNA or wild-type TRV infected tissue extract according to 4.2.2.2. At 10 dpi, one root system from a virus-free plant and three root systems from TRV-GNA and TRV-fsGNA infected plants were washed free of sand. Tissue prints were performed according to method 2.2.24. At 21 dpi, the root system of a TRV-GNA and TRV-ΔGFP infected plant were collected and washed for tissue printing as above.

4.2.3.5 TRV-GNA stability study

The following experiment was designed to determine the stability of the GNA sequence, inserted in TRV-GNA, after serial passages through *N.benthamiana* plants.

Ten *N. benthamiana* plants were coinoculated with TRV/K20/GNA transcript and total RNA (see section 4.2.1). Total root mass was collected from each plant at 7 dpi and the tissue extract analyzed by western blotting. Infected root sap (first passage) was then used to inoculate ten *N. benthamiana* plants. Root tissue from this experiment was collected at 14 dpi and analyzed as above. Infected root extracts were used as second passage inoculum for the consecutive experiment.

This process was repeated for four serial passages. Third and fourth passage root extract was collected at 11 dpi and 9 dpi respectively. For comparison 15 µl of inoculum from each experiment was analyzed by western blotting and probed with anti-GNA or anti-CP antibodies.

4.3 Results

4.3.1 Detection of virally expressed foreign proteins

In this first experiment western blotting was used to detect virally expressed proteins in tissue extracts from *N. bethamiana* plants inoculated with either TRV-GNA, TRVfsGNA or TRV-ΔGFP transcript RNA. TRV induced lesions on the inoculated or systemically infected leaves were not easily detected by eye, therefore tissue samples were screened by western blotting according to the pattern of systemic GFP expression observed in Chapter 3. The study leaf was collected for the following experiments according to previous observations.

Root and leaf samples of TRV-GNA transcript inoculated *N. bethamiana* plants were collected at 10 dpi. Figure 4.1 shows that CP and GNA (molecular weights of 27 and 12.5 kDa respectively) were detected by western blotting after probing with anti-CP (Blot A) and anti-GNA (provided by Durham University) (Blot B). Only two of the six inoculated plants were systemically infected by TRV-GNA transcript. However, all plants inoculated with first passage inoculum collected from the previous experiment produced a systemic infection in leaves (Blot C) and roots (Blot D) at 7 dpi (see Figure 4.1). GNA was not detected in mock-inoculated *N. bethamiana* plants. Anti-GNA2 antibodies (supplied by Axis Genetics) did not detect GNA expressed by the virus vector, but did react with the recombinant GNA purified from *E. coli.*

Root samples from TRV-fsGNA transcript inoculated *N. bethamiana* plants were analyzed by western blotting at 9 dpi and compared to root extracts collected from TRV-GNA inoculated plants (7 dpi) (see Figure 4.2). GNA and CP were detected in four of the six TRV-GNA inoculated plants. CP detected in lane 2 was probably due to leakage during gel loading as GNA was not detected. CP was detected in two of six TRV-fsGNA inoculated plants, but the fsGNA protein was not detected in these samples by the GNA-specific antibody.

A BLAST database search was carried out for the amino acid sequence of the frame-shift GNA peptide after position 249. Analysis by sequence alignment indicated that the nearest match for the frameshift amino acid sequence had less than 37 % identity to a

hypothetical *Caenorhabditis elegans* (nematode) protein Y25C1A.3, followed by a hypothetical glycine-rich protein isolated from Mesorhi (reference NP 102359.1).

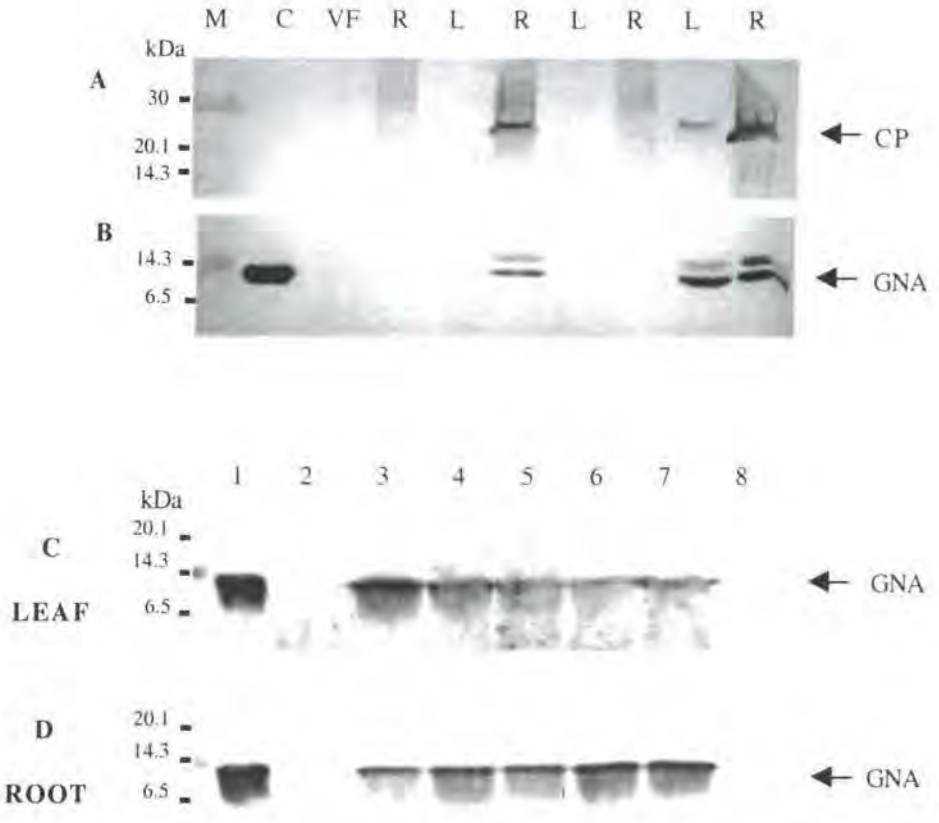


Figure 4.1 Western blot of *N. benthamiana* plants inoculated with TRV-GNA. Lanes were loaded with extracts of leaf or root extract. Panel A and B, shows samples harvested at 10 dpi from transcript inoculated plants. M denotes protein size markers. C, 250 ng purified GNA protein. VF, virus-free tissue extract. Lanes 4-10, plant root (R) and 3 leaf (L); plant 4 R and L; plant 5 R; plant 6 L and R. Panel A was probed with CP-specific antibodies. Panel B was probed with GNA-specific antibodies. Panel C and D, show samples harvested at 7 dpi from first passage inoculated plants. Inoculum was root extract originating from plant 6. Lane 1, 500 ng purified GNA protein, lane 2, virus-free tissue extract, lanes 3-8, plants 1-6. Blot presented in panel C (leaf extracts) and panel D (root extracts) were probed with GNA-specific antibodies. Root extracts were prepared from the complete root system excluding the hypocotyl.

Figure 4.2 Western blot of *N. benthamiana* plants inoculated with transcript TRV-GNA and TRV-fsGNA. Lanes were loaded with equal volumes of root extract. Lanes 1-6, TRV-GNA infected plants at 7 dpi. Lanes 7-12, TRV-fsGNA inoculated plants at 9 dpi. Panel A and B was probed with CP-specific antibodies and GNA-specific antibodies respectively.

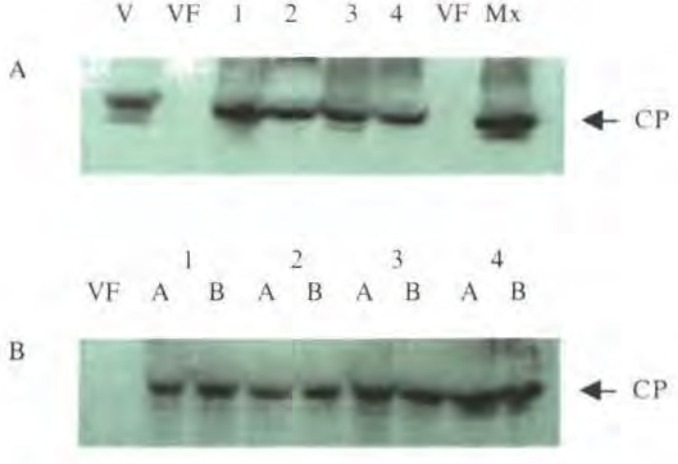


Figure 4.3 Western blots of extracts of *N. benthamiana* plants inoculated with TRV-ΔGFP, a TRV virus expression vector with an empty expression cassette. Panel A shows first passage extracts (transcript inoculated plants). Lanes V, TRV infected extract. VF, virus-free extract. Lanes 1 – 4, TRV-ΔGFP infected plants at 7 dpi. Mx, mix of tissue extract from TRV and TRV-ΔGFP infected plants. Panel B shows inoculum viability of first passage inoculum (tissue extracts from plants 1-4 shown in panel A). A and B represent duplicate infections. VF, virus-free extract. Blots probed with anti-CP antibodies.

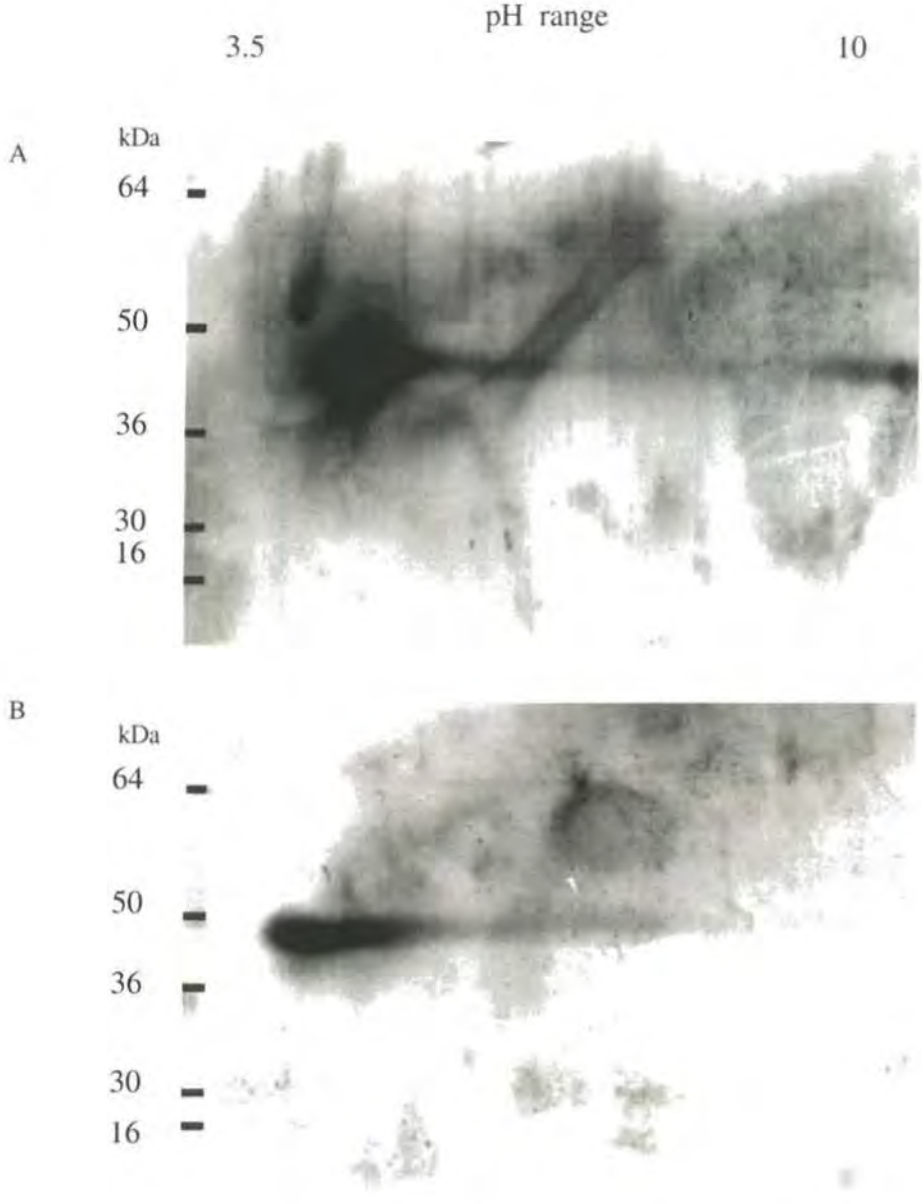


Figure 4.4 Non-denaturing two dimensional, isoelectric focusing blot of GNA expressed by the TRV vector in systemically infected leaves and roots at 9 dpi. Extracts were separated using ampholytes ranging from pH 3.5 to 10. Column was loaded with leaf (panel A) and root (panel B) samples respectively. Blots were probed with GNA-specific antibodies.

Roots from TRV-ΔGFP transcript inoculated *N. bethamiana* plants were analyzed by western blotting at 7 dpi. Figure 4.3 Blot A indicated that CP was detected in all root samples. CP from a mixture of TRV-GNA and TRV-ΔGFP root extract was resolved into a single band of 27 kDa (Figure 4.3 Blot A, lane 8). Figure 4.3 Blot B indicated that CP was detected in systemically infected roots after inoculation by TRV-ΔGFP first passage inoculum.

Detection of virus CP or GNA in the sink leaves above the inoculated leaf indicates that the virus had spread from the inoculated leaf producing a systemic infection. Infected tissue extract (first passage) collected from TRV-GNA and TRV-ΔGFP infected plants were able to produce infections and therefore were viable sources of inoculum for subsequent experiments.

4.3.2 Tetrameric GNA was detected in TRV-GNA infected tissue extracts

In the previous experiments GNA was detected after SDS-PAGE as a protein with the M*r* of approximately 12.5 kDa. To determine whether tetrameric GNA was present in systemically infected plants, tissue extracts were analyzed by 2D IEF using ampholytes ranging from pH 3.5 to 10, in non-denaturing conditions. Figure 4.4 presents blots of leaf and root extracts sampled from TRV-GNA transcript inoculated plants at 9 dpi. The blots indicate that a single large protein spot of approximately 50 kDa was detected in both the leaves (Blot A) and roots (Blot B) after probing with anti-GNA antibody.

4.3.3 Time course observations for TRV-GNA in *N. benthaminana* plants

A series of time course experiments were performed to assess GNA accummulation in *N. benthamiana* leaf and root tissue. The first time course sampled the study leaf and roots (not including hypocotyl) of 16 plants at three day intervals for a period of 24 days. The blot in Figure 4.5 indicates that systemic CP and GNA were first detected in the study leaf at 3 dpi and roots at 6 dpi. The signal for CP and GNA in both tissues was the greatest at approximately 6 dpi and this signal then gradually decreased for a further 21 days until the last sample date (24 dpi). Some differences in protein accumulation was evident from the blots shown in Figure 4.5. In leaf tissue, at 18 dpi the signal for GNA

(Blot A) was decreased more rapidly than CP (Blot B). Greater levels of GNA may be expressed in root compared to leaf tissue. In the roots, CP and GNA accumulation was greatest at 6 dpi (Blot C and D respectively), both proteins then gradually decreased at a similar rate over the next 16 days.

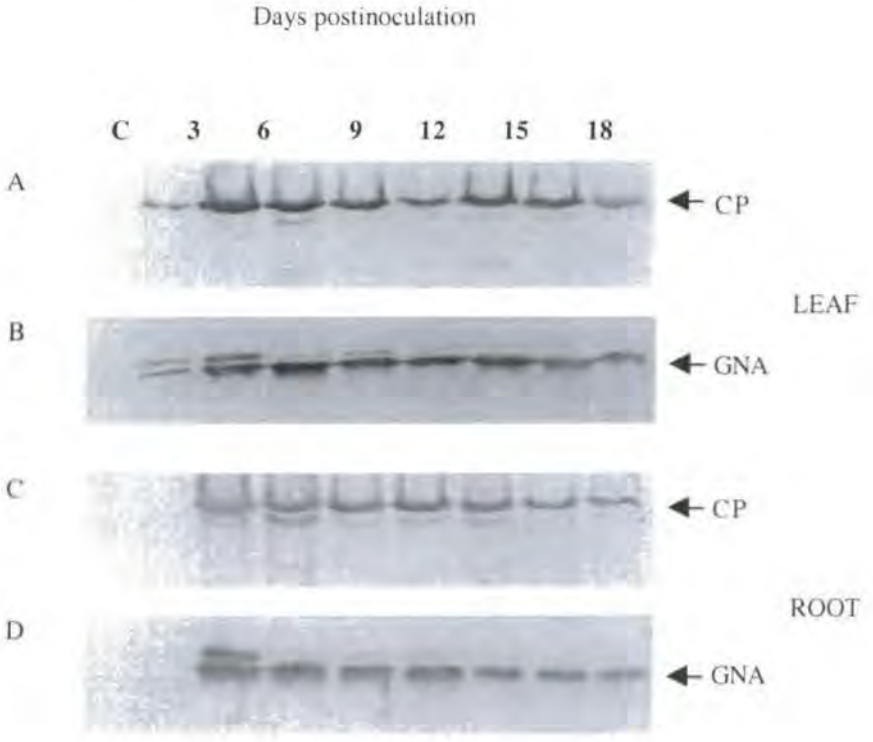


Figure 4.5 Time course of synthesis of CP and GNA in TRV-GNA infected *N. benthamiana* plants. Two systemically infected leaves (panel A and B) and the complete root system (panel C and D) were sampled. Lanes were loaded with equal volumes of sap. VF, virus-free extract. Time course samples are shown as 3-24 days postinoculation. Blots shown in panels A and C were probed with CP-specific antibodies. Blots shown in panels B and D were probed with GNA-specific antibodies.

Figure 4.6 Virus-free *N. benthamiana* plants grown in sieved sand, in a 30 ml pot (A) at 27 days growth and (B) approximately 40 day growth.

4.3.3.1 GNA is expressed in small *N. benthamiana* plants grown in sand

Time course experiments were also performed on small *N. benthamiana* plants that had been propagated in sand, in 30 ml pots (see Figure 4.6). This background information was required for the nematode bioassay experiments described in Chapter 5, which were to be routinely performed on these smaller plants.

The second time course confirmed that GNA was expressed by TRV-GNA in the roots of small *N. benthamiana* plants. Root systems of TRV-GNA infected plants were collected daily between 2 dpi and 23 dpi and analyzed by western blotting. The root blots presented in Figure 4.7 (panels A and B) indicate that approximately 25 ng GNA was detected in 12 µl tissue:laemmli (equivalent to 6 µl of root extract) between 4 dpi to 23 dpi (the last sample date). This latter observation indicated that the time course of GNA expression in the smaller plants was comparable to that recorded in plants in the previous time course experiment (see section 4.3.3) which were grown in compost.

The third time course experiment was repeated to determine whether the higher temperatures (26 - 28 °C) required for the growth and development of the root knot nematode would affect on the replication of TRV-GNA (a temperate virus) and the accumulation of GNA in the *N. benthamiana* root system. This time course was repeated according to the previous experiment, except that after a period of 8 days the TRV-GNA

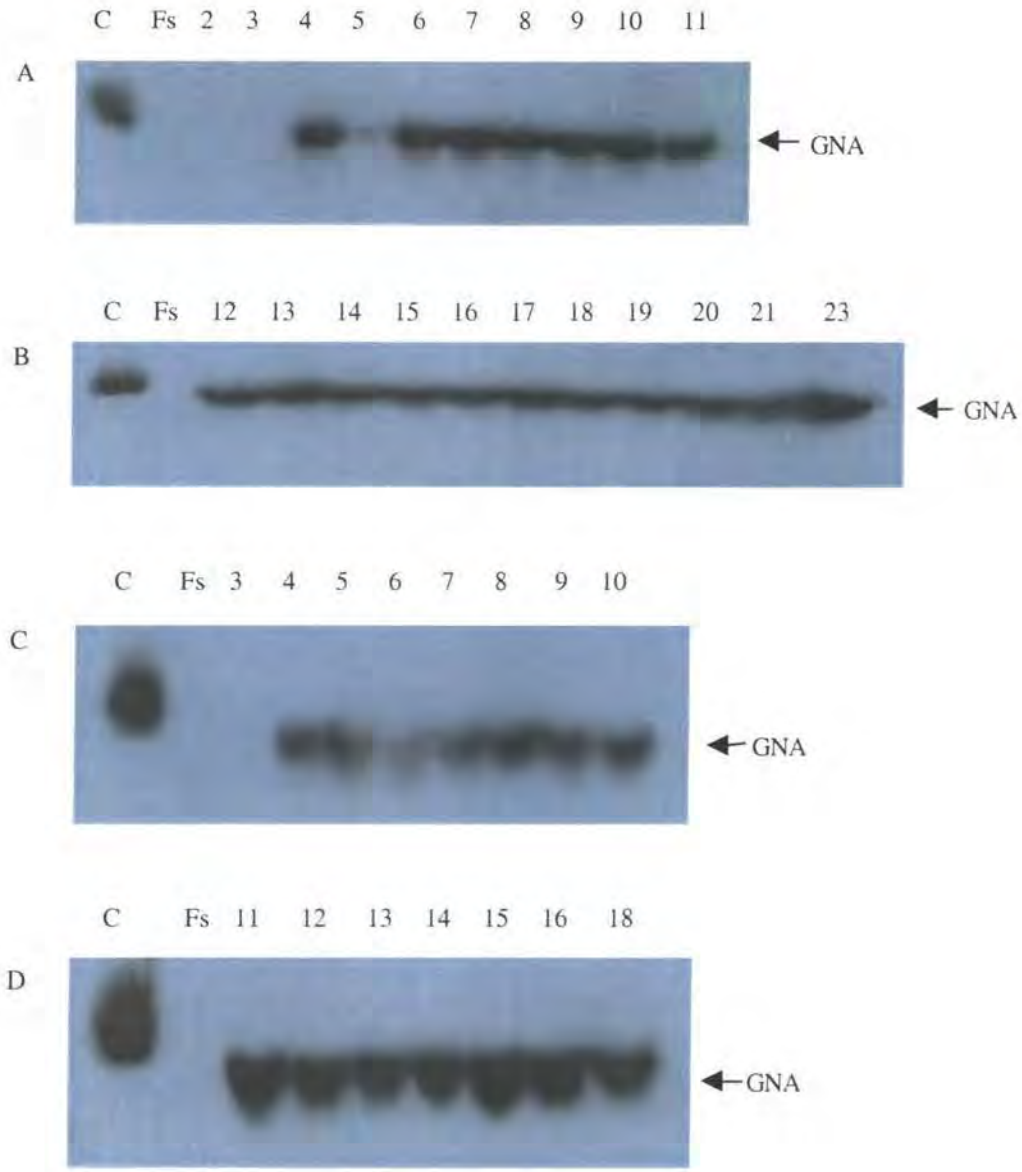


Figure 4.7 Western blot of time course of GNA expression by the TRV-GNA expression vector in *N. benthamiana* plants (grown in sand in 30 ml pots). Lanes were loaded with equal volumes of root extract. C, 25 ng purified GNA protein. Fs, root extract from a TRV-fsGNA infected plant. Panel A shows infected root samples at 2-11 dpi. Panel B shows the infected root samples at 12-23 dpi. Plants were transferred from a temperate glasshouse to heated glasshouse at 8 dpi. Lanes were loaded with equal volumes of tissue extract. C, 50 ng purified GNA protein. Fs, root extract from a plant inoculated with TRV-fsGNA. Panel C shows the infected root samples at 3-10 dpi. Panel D shows the infected root samples at 11-23 dpi. Blots were probed with GNA-specific antibodies.

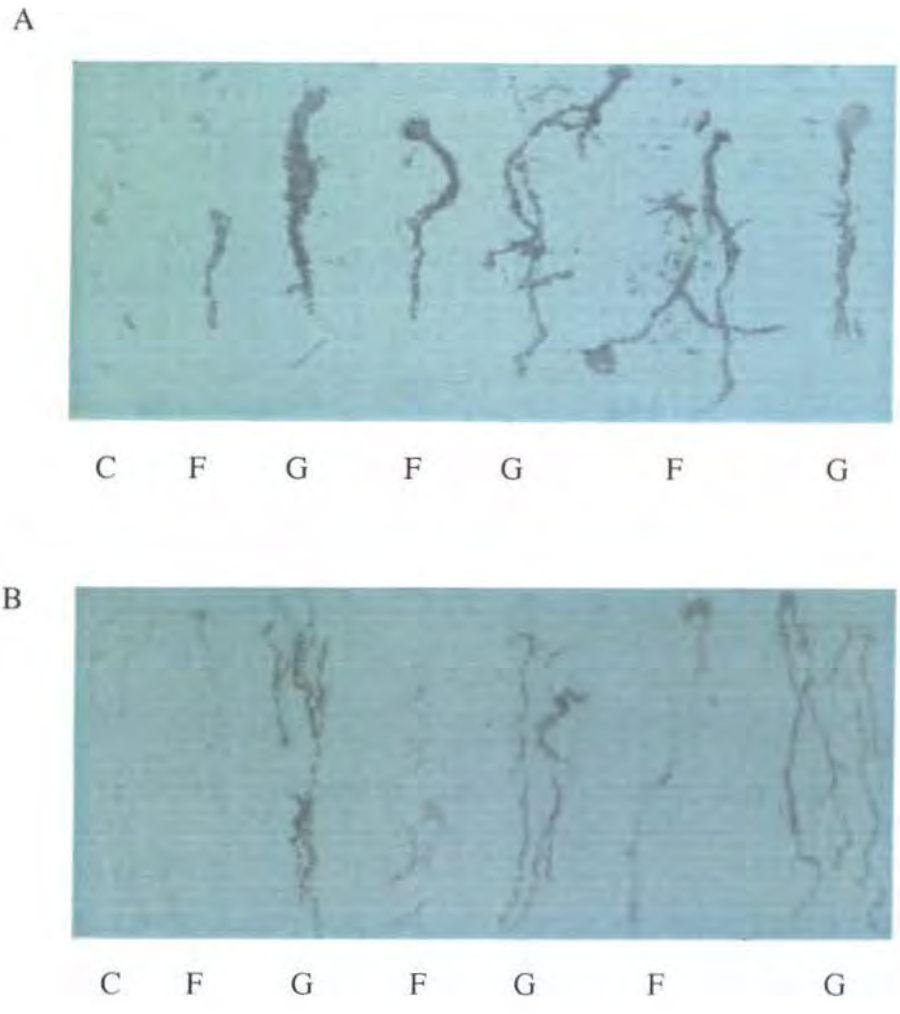


Figure 4.8 Tissue print of *N. benthamiana* roots infected with TRV-GNA and TRV-fsGNA at 10 dpi. Plants were grown from seedlings in sand, in 30 ml pots. Roots from mock- inoculated plants (C), TRV-fsGNA infected plants (F) and TRV-GNA infected plants. Blots were probed with anti-cp antibodies (panel A) or anti-GNA antibodies (panel B).

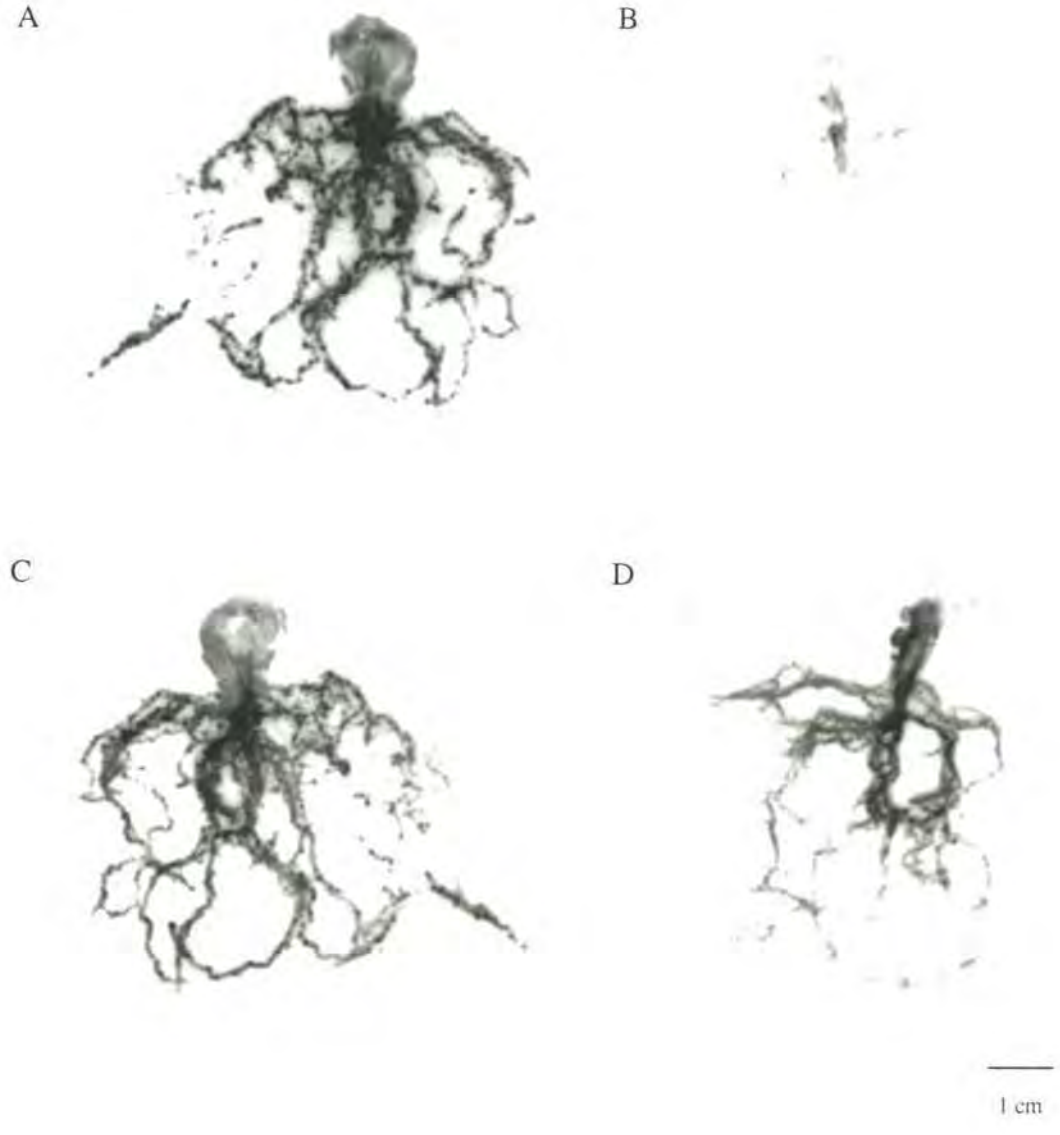


Figure 4.9 Tissue print of *N. benthamiana* roots. Plant infected with TRV-GNA at 21 dpi and probed with (A) GNA-specific antibodies and (C) CP-specific antibodies. Wild-type TRV infected plant probed with (B) GNA-specific antibodies and (D) CP-specific antibodies.

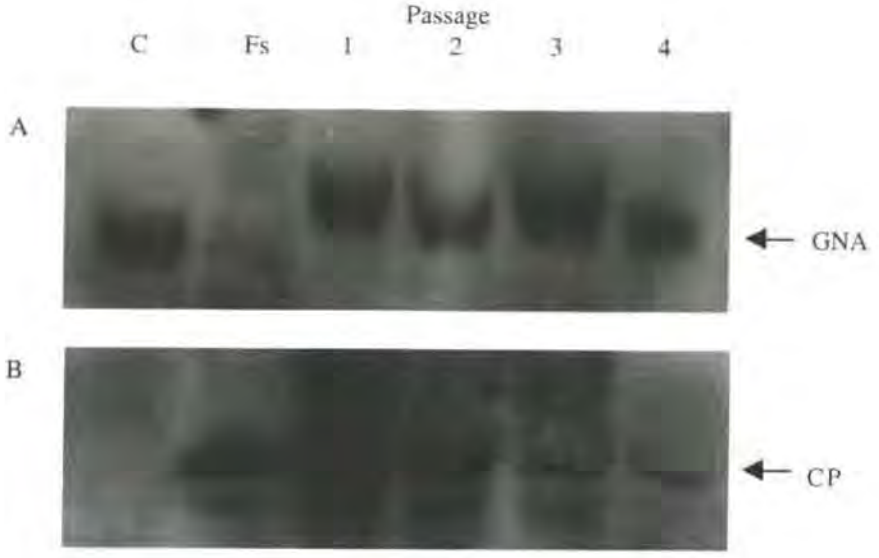


Figure 4.10 Western blot showing GNA expression by TRV vector over four serial passages in *N. benthamiana* plants. Lanes were loaded with equal volumes of root extract. Lane C is 50 ng purified GNA protein. Lane Fs is root extract from a TRV-fsGNA inoculated plant. Lane 1 is first passage (7 dpi); lane 2 is second passage (14 dpi); lane 3 is third passage (11 dpi) and lane 4 is fourth passage (9 dpi) tissue extract. Panel A and B were probed with GNA-specific and CP-specific antibodies respectively.

infected *N. benthamiana* plants were transferred to a heated glasshouse. Two root samples were collected daily between 3 dpi and 18 dpi and analyzed by western blotting. The root blots presented in Figure 4.7 (panels C and D) indicates that approximately 50 ng GNA was detected in 6 µl of root extract (12 µl tissue:laemmli) between 4 dpi to 18 dpi (the last sample date) and therefore demonstrating that GNA expression by TRV-GNA was not affected by the higher temperature.

4.3.3.2 TRV-GNA expresses GNA throughout the whole root system of infected *N. benthamiana* plants

Tissue printing was used to vizualize the distribution of virally expressed proteins in the root system of small *N. benthamiana* plants grown in sieved sand, in 30 ml pots. Figure 4.8 presents the tissue prints of a mock-inoculated (virus-free) root system

compared to roots infected by TRV-GNA or TRV-fsGNA, at 10 dpi, after probing with anti-CP antibodies (panel A) anti-GNA antibodies (panel B). Figure 4.9 presents the tissue prints of a root system infected by TRV-GNA (panel C and E) and wild-type TRV (panel D and F) at 21 dpi, after probing with either anti-GNA or anti-CP primary antibody (top and bottom panels respectively). The results indicate that anti-CP antibodies can be used to identify roots infected by the virus in both TRV-fsGNA and TRV-GNA infected plants. In contrast, the anti-GNA antibody is highly specific to GNA, although a greater background signal is visible in the TRV-fsGNA infected roots compared to the virus-free roots.

4.3.4 Long term stability of the TRV-GNA construct

To assess the long-term stability of the TRV-GNA virus construct *N. benthamiana* plants were co-inoculated with TRV/K20/GNA transcript and total RNA, and then serially with first to third passage inoculum (see section 4.2.1). Western blotting was used to identify TRV-GNA infected roots, which were then used as inoculum for the following experiment. The root blots presented in Figure 4.10 show that GNA (panel A) and CP (panel B) was detected in root tissue sampled from the first to fourth passage inoculum (7 dpi, 14 dpi, 11 dpi and 9 dpi respectively).

4.4 Discussion

4.4.1 Description of the TRV-GNA virus-based vector and derived mutant clones

The sequence of snowdrop lectin LECGNA2 (accession number M55556) was first reported by Van Damme *et al.*, (1991a). The sequence of the PCR amplified GNA gene used for this research is presented in Figure 2.1. Two additional restriction sites were introduced into the LECGNA2 gene by PCR mutagenesis to enable directional cloning of the GNA gene into the TRV virus vector. An *Avr*II site was introduced at position 21 (altering the amino acid from alanine to proline) and a *Kpn*I site was introduced at position 497, downstream of the translation termination codon (position 492) (for subcloning of GNA into the TRV gene expression vector see section 2.2.2 and Figure 2.2). Translation of the cloned GNA sequence indicated that alanine would be incorporated instead of valine at position 85 in the mature peptide. A second base substitution in the C-terminal peptide of GNA (glutamic acid to valine at position 489) was also identified, but this region of the peptide is removed postranslationally in the rough ER (Van Damme and Peumans, 1996).

Two TRV virus mutant clones were also produced for this study. For the TRV-fsGNA clone, a frame-shift was introduced the GNA sequence at position 254 by digesting with *Sph*I followed by treatment with Klenow polymerase (see section 2.4.1). For the TRV-ΔGFP clone, the TRV-GFPc clone was digested with *Kpn*1 and *Nhe*I, to remove the GFP gene. No foreign genes were expressed from this mutant (see section 2.4.2).

4.4.2 Characterization of GNA expression in *N. benthamiana* by TRV-GNA

The snowdrop lectin GNA has previously been expressed in tomato and potato by *Agrobacterium* transformation indicating that this protein could be expressed in alternative host plant species. The experiments undertaken in this chapter were designed to demonstrate that the cloned GNA sequence could be be expressed by the TRV expression vector, in *N. benthamiana* plants and, despite alterations in the primary amino acid sequence, be recognized by anti-GNA antibodies.

The first set of experiments in this study were carried out to show that the TRV-GNA (Figure 4.1), TRV-fsGNA (Figure 4.2) and TRV-ΔGFP (Figure 4.3) transcripts,

when co-inoculated with TRV RNA1, were biologically active and capable of producing systemic infections in *N. benthamiana*. Blots probed with anti-CP antibody did accurately detect coat protein in leaf and root extracts infected by TRV-GNA, TRV-fsGNA TRV-ΔGFP. GNA expression by TRV-GNA was detected by the anti-GNA antibody supplied by Durham University. But this antiboody did not recognize the frameshift GNA protein (Figure 4.2). This latter observation may be due to several reasons, for example: 1) loss of antigenicity due to the change or absence of surface epitopes of the truncated GNA protein; 2) the protein was not present due to the suppression of virus expression, or 3) the truncated protein was degraded. Sequence analysis of the frameshift GNA sequence was presented in Figure 2.1 B) and revealed a 8 base deletion, which produced a reading frame-shift after position 248 and the creation of a premature termination codon (TGA) at position 369.

Differences in specificity of the polyclonal anti-GNA antibodies supplied by Durham University and Axis Genetics could be related to several factors like small alterations in protein folding associated with the expression vector (personal communication by Dr Richard Napier, HRI) or method of antibody production. The first distinction between the Axis Genetics and Durham Universities antibodies were that the former was raised to the recombinant bacterial GNA protein whereas the latter was raised against partially denatured native (tetrameric) GNA isolated from plant tissue. It is therefore possible that the Axis Genetics antibodies were highly specific to recombinant bacterial GNA. Alternatively, if the recombinant GNA were a preproprotein then the C-terminal peptide sequence, which is an epitope, would have provided additional antigenicity (personal communication by Dr John Gatehouse).

4.4.3 Truncated GNA is hypothetical merolectin

In an attempt to understand the structure of the truncated GNA molecule the amino acid sequence of GNA and fsGNA were aligned according to the subdomain regions described by Hester *et al.*, (1996). Figure 4.9 indicates that the first 54 amino acid residues of the truncated GNA protein were identical to GNA. These amino acid residues were equivalent to the first (innermost) β-sheet of subdomain I and II, and also to the

Figure 4.11 Sequence alignment of the subdomain regions for GNA (shown in blue, is adapted from Hester *et al.*, 1996). Subdomains I, II and III are flat four stranded, antiparallel β-sheets with individual strands labelled β1-12 (shown underlined). Conserved residues contributing the polar surface of the 3 mannose binding-sites are highlighted in bold (Hester *et al.*, 1996). The translated amino acid sequence of GNA that is shared by the truncated protein is shown in black. To illustrate the change in the amino acid residues after the frame-shift the GNA is shown in blue and the changes in the truncated protein shown in red. Uncoded residues after the premature termination codon are represented as *.

Subdomain		Strand 1	Strand 2	Strand 3	Strand 4
		β1	β10	β11	β12
I	GNA	DNILYS	GNYVCILQK DRN	VVIY-GTDR	– – WATGTHTG
	Amino acid	1 6	82		109
	fsGNA	DNILYS	ITCASYRRL GML		
		β2	β7	β8	β9
II	GNA	GETLST	RSCFLSMQTD	GNLVVYNPSNKPI	WASNT – GGQN
	Amino acid	7 12	50		81
	fsGNA	GETLST	RSCFL ILLMG TSWC TTHR INRFG QATLL AKMG		
		β3	β4	β5	β6
III	GNA	GEFLNY	GSFVFIMQEDCNLVLYD – VDK	PI WATNT – GGLS	
	Amino acid	13 18	19		49
	FsGNA	GEFLNY	GSFVFIMQEDCNLVLYD – VDK	PI WATNT – GGLS	

residues that would form the anti-parallel four-stranded β-sheets of subdomain III (G13 to G47, see Figure 4.11). If the fsGNA peptide folded to form subdomain III, then formation of a disulphide bond between cysteine residues C29 and C52 might stabilize its stucture. Consequently, formation of subdomain III would potentially provide the truncated GNA protein with a single mannose-binding site. Interestingly, amino acid analysis has shown that the mannose-binding site on subdomain III is the most highly conserved of the three binding sites within the superfamily (Barre *et al.*, 1996). No match for the frameshift GNA amino acid sequence was found in the BLAST database, this suggested that nonsense amino acid coding was translated after the frameshift. The fsGNA polypeptide was also 16 residues shorter than the GNA peptide. These latter observations indicate that the truncated peptide would lack the C-terminal region (residues 99 - 109) that is necessary for strand exchange, dimer formation and docking of further mannose units.

Although it is not possible to ascertain from these observations whether the lack of recognition by the anti-GNA antibody is related to the C-terminal region, subdomain I and or subdomain II. The observations suggest that if the truncated GNA protein is stably expressed by the TRV-fsGNA expresson vector, then it might be described as a merolectin (see section 4.1) as it is probably monomeric, with some affinity for mannose residues.

4.4.4 Virally expressed GNA assembles into its tetameric form

To characterize the GNA expressed by TRV-GNA, tissue extracts collected from TRV-GNA infected plants were analyzed in both denaturing (SDS-PAGE) and non-denaturing conditions (2D isoelectric focusing) and the resulting blots probed with anti-GNA antibodies. In denaturing conditions SDS-PAGE fractionated a monomeric polypeptide with the molecular mass of 12.5 kDa (Figure 4.1). A large protein with the molecular mass of approximately 50 kDa was detected after 2D IEF (see Figure 4.4). Similar results have been observed after analysis of crude bulb extracts (Van Damme *et al.*, 1987a, 1991a,c). These results suggest that the cloned GNA used in this study was successfully expressed *in planta* by the TRV gene expression vector and that the GNA peptides assemble into the tetrameric molecule.

4.4.5 GNA is stably maintained in the TRV virus expression vector

A primary aim of this study was to establish whether the GNA sequence was stably maintained in the virus genome like the GFP sequence. The results from this experiment indicate that, like GFP (see section 3.3.3), GNA and CP were detected by denaturing western blotting after four serial passages (Figure 4.10, Blot A and B respectively). This suggests that the GNA ORF was not affected by recombination over this period (MacFarlane and Popovich, 2000) and that the TRV-GNA virus vector would be a viable and durable source of GNA for further experiments. The effect of serial passages on the formation of additional RNA species by RT-PCR products was not undertaken for these samples due to limited time. However, the observations from this experiment may support the hypothesis that recombination is less likely to occur between distantly related sequences (Lai 1992).

4.4.6 Virally expressed GNA rapidly accumulates in leaves and roots

The main aim of the following time course experiments was to determine the stability of accumulated GNA in a systemic leaf (the study leaf which was designated as the third sink leaf above the inoculated leaf) and roots and their potential application as host plants for aphid and nematode pests in a bioassay experiment. Data from the GFP expression time course experiments (see section 3.3.1) was used as a reference for the collection of leaf and root material for the present TRV-GNA study. Three time course experiments were carried out.

The aim of the first TRV-GNA time course experiment was to assess GNA accumulation in the study leaf and total root mass of a systemically infected *N. benthamiana* plant grown in compost. Tissue samples were collected at three-day intervals and examined by western blotting. The blots in Figure 4.5 show that both CP and GNA were detected in the leaves from 3 dpi and, due to the sampling regime used in this experiment, were first detected in the roots at 6 dpi. The blots show that there is good correlation between the level of GNA and CP signal detected in each tissue type until 24 dpi (last sample date) and their decline over this period. Detection of GNA in the study leaf during the first time course experiment combined with the previous observations of

GFP expression in this leaf (section 3.3.1) suggested that the systemically infected leaf could be subsequently used as the platform leaf for aphid clip-cage experiment. For aphid inoculation purposes the systemically infected leaf could be identified in the canopy because of very slight leaf crinkling.

In contrast to the time course for TRV-GFP, GFP was only detected up to 18 dpi and CP to 22 dpi (section 3.3.5). This result confirms the speculation made in the discusion of chapter 3 that the decline of the signal detected by western blotting is dependent on the nature of the expressed protein. In the case of GNA, this protein might be expected to have increased longevity compared to GFP because of its role as a storage protein. However, the detection of CP after 24 dpi may also suggest that recovery from the TRV-GNA infection by *N. benthamiana* was inhibited by the presence of GNA.

Detection of GNA in the roots until 24 dpi combined with the previous observations of GFP expression in roots (see Chapter 3) suggested that TRV-GNA infected roots could be used for nematode-feeding experiments. However, as nematode bioassays were to be routinely carried out on small *N. benthamiana* plants grown in sieved sand (grain size 1000 – 500 μm) which would provide a standardized substrate for nematode movement and also a smaller mass of roots for processing and root gall counts (see Chapter 5) time course experiments were repeated. The second time course indicated that approximately 25 ng GNA was detected in the roots of small *N. benthamiana* plants between 4 dpi to 23 dpi (see blots A and B in Figure 4.7). However, unlike the first time course experiment, the level of accumulated GNA did not decline over the time course period. The aim of the third time course was to ensure that GNA accumulation and stability was not affected by the higher temperatures required for root knot nematode development. The second time course experiment was repeated except that plants were transferred from an unregulated glasshouse to a heated glasshouse after 8 dpi, which allowed the systemic viral infection to develop under temperate conditions but also accommodated the higher temperature requirement of the root knot nematode. The blots shown in Figure 4.7 (C and D) indicate that approximately 50 ng of GNA was detected between 4 dpi and 18 dpi, which suggested that the accumulation of GNA was not affected by a warmer environment.

Differences between the level of GNA detected in the root blots of the second and third time course were also observed. These differences may originate from variation of virus particles present in aliquots of inoculum, the plant population used or external factors like seasonal variation. It was also noted that decline of the GNA signal over the time course was dependent on the level of accumulated GNA at the initial stage of the virus infection. However, these experiments demonstrated that GNA was expressed in roots for a period of a minimum of 15 to 20 days, which was an interval that would be inclusive of the 10 to 14 days required for visible root gall formation by the developing root knot nematode (see Chapter 5). The data from these time course experiments therefore suggests that the period of GNA expression in roots would accommodate a nematode bioassay.

In Chapter 3, TRV-GFP was found to express GFP in uniform, solid regions in infected *N. benthamiana* roots (see section 3.3.5). This uniform pattern of protein expression was thought to be an important consideration for a pathogen bioassay as it would provide a standardized area of root available for the feeding nematodes.

Tissue prints of virus infected roots were collected from either TRV-GNA (10 dpi or 21 dpi) or TRV-fsGNA (10 dpi), wild-type TRV infections (21 dpi), (Figure 4.8 and 4.9) allowed visualization of CP and GNA spread through the whole length of an infected roots. This observation supported the application of small *N. benthamiana* plants, grown in 30 ml pots, in nematode bioassays. The root squashes also demonstrate that the spread of the TRV virus expression vector can be distinguished using either the anti-CP antibody or the anti-GNA antibodies.

4.4.7 Conclusion

The results from this study demonstrate that the TRV expression vector could provide a versatile delivery agent for both leaf and root feeding pathogens.

1) The virally expressed GNA peptide assembles to form the 50 kDa tetrameric GNA molecule. Anti-GNA antibodies could detect both monomeric and tetrameric GNA molecules.

2) Mutant clones of the TRV or TRV-GNA could be produced (TRV-GFP and TRV-fsGNA respectively) which could be detected in tissue extracts by anti-CP antibodies. The mutant TRV clones would serve as comparison treatments for the future bioassays.

3) Virally expressed proteins were detected in the study leaf and root tissue until 24 dpi (last sample). A period of time that would accommodate both aphid and nematode bioassays and therefore ensure that feeding pathogens were exposed to a rich source of the pesticidal protein. Tissue prints of infected roots confirmed that GNA and CP were expressed through the whole length of the roots system at 10 dpi and 21 dpi

Chapter 5

Effect of snowdrop lectin on root gall production by root-knot nematodes (*Meloidogyne* spp.)

5.1 Introduction

Management of important endoparasitic nematodes pests are limited to crop rotations or the application of toxic chemicals nematicides that have undesirable environmental and secondary effects. Utilization of plant derived proteins that have toxic effects (lectins and enzyme inhibitors) is one of many

strategies being researched in the effort to engineer nematode resistance. For example, antisense and gene co-suppression, cytotoxic genes, antibodies and cloning of plant gene promoters that function within the feeding site (Burrows, 1996). Transgenic resistance expressing anti-nematode proteins would therefore offer the potential of generic defence against a wide range of nematodes while reducing chemical input. Research in to rapid screening of gene products with nematicidal properties could accelerate this process and reduce the associated labour costs.

5.1.2 Aim

The main objective of this study was to assess the effectiveness of the TRV virus expression system as a rapid method of screening primary gene products, *in planta*, against nematode pathogens. To accomplish the bioassays 'rapidly' the bioassays focus specifically on galling as criteria for measuring the response of invading juvenile root knot nematodes (J2).

5.2 Materials and Methods

5.2.1 Nematodes

Nematode populations were reared on tomato (*Lycopersicon esculentum* Mill cv. Moneymaker) planted in sand and fertilized by nutrient solution H (1 l contains 0.75 g Solufeed H (Solufeed™), 0.4 M KNO_3 and 0.26 M $Ca(NO_3)_2$). Glasshouse conditions were set at 28 °C with 10 h light, although subtropical temperatures (16 °C) were noted periodically; these suboptimal conditions resulted in a delay in nematode development and egg production which caused problems regarding the timing of the bioassay experiment inoculation. Therefore, roots were individually inspected for mature females and the presence of egg masses before harvesting. Experiments were set up weekly to compensate for this variation. Infective juveniles (J2) were extracted from either tomato plants maintained in hydroponic culture (*M. incognita*). Or, by the Seinhorst-mist technique (Barker 1985) where detached tomato root were placed in funnels supported in 50 ml tubes for specimen collection (*M. javanica*). Automated misting occurred at 5-minute intervals. After collection, all juvenile stages (J2) were stored in small volumes of tap water at 4 °C.

5.2.1.1 Specific nematode lines were used for each bioassay

Bioassays 1 to 6 – *N. benthamiana* roots were inoculated with *Meloidogyne spp.* (a population of *M. incognita* L27 found to be contaminated by *M. javanica,* Mark Phillips personal communication).

Bioassays 7 to 10 – *N. benthamiana* roots were inoculated with *M. javanica* Crete line 17 (virulent against the Mi gene, Mark Phillips, personal communication).

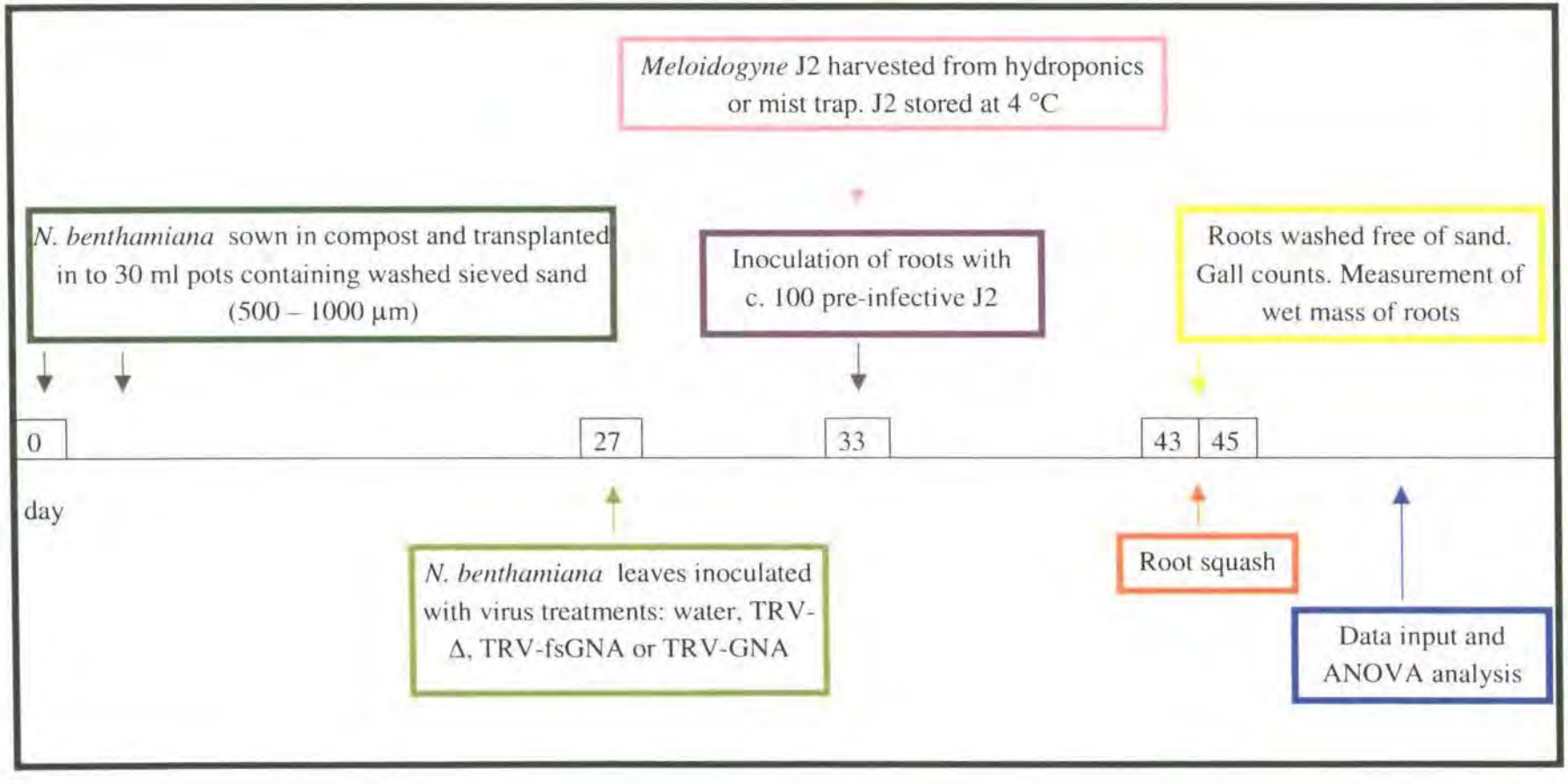
5.2.2 Standard nematode infection bioassay method

The standard nematode infection bioassay is described schematically in Figure 5.1.

5.2.2.1 Plant propagation

A) *N. benthamiana* seedlings were sown in compost and transferred after 10 to 12 days into 30 ml experimental pots containing washed, sieved sand (500 - 1000 µm grain

Figure 5.1 Schematic representation of the rapid *in planta* nematode bioassay experiment showing approximate time taken in days



diameter) according to the method described by MacCullock (1990). Seedlings were then grown at 21 °C and watered when required with nutrient solution H.

B) Approximately 6 days after sowing *L. esculentum* seedlings (tomato, cv Moneymaker) were transferred to sieved sand and grown for a further 11 days until the first true leaves were visible.

5.2.2.2 Plant inoculation

A) The standard virus inoculation protocol described in Section 2.19 was followed for this study. For each bioassay, twelve *N. benthamiana* plants (including two reserve plants) were inoculated at 27 to 29 days old (three true leaves growth) with one of the following treatments; TRV-GNA, TRV-fsGNA, TRV-ΔGFP or water (mock-inoculated control). The progress of the virus infection was assessed by comparing symptom development to TRV-GFP inoculated plants. *N. benthamiana* plants were inoculated with nematodes 7 to 9 days post virus inoculation.

B) Cotyledons of tomato seedlings were inoculated with the plant treatments (see above) after 17 days of growth.

5.2.2.3 Nematode inoculation

A) Prior to experimentation, stored pre-infective second stage juvenile nematodes (J2) were placed on tissue (Kleenex) supported by a sieve to separate out motile and viable infective individuals. Counts of J2 per 100 µl water were made using a Zeiss binocular microscope. Pre-infective J2 were inoculated onto partially exposed roots as 100 J2s per volume of water per plant. Bioassays were transferred to a greenhouse set at 22 - 28 °C (10 hours light) and watered sparingly with nutrient H solution to avoid water-logging.

B) Tomato roots were inoculated with 300 J2s per tomato plant 12 - 14 days post viral inoculation. Tomato bioassays were performed alongside the *N. benthamiana* bioassays.

5.2.2.4 Assessment of nematode galling

'Galling' was used as a visible index to measure nematode establishment. Galls (induced feeding site resulting from successful invasion of the root by the infective

juvenile (J2) stage) were identified as discrete, uniform nodules, wider than root diameter and situated at any point along the root length depending on root age.

5.2.2.5 Root tissue samples

A) *N. benthamiana* roots samples were harvested between 10 to 11 days post nematode inoculation, washed free of sand and then placed in petri dishes. Galls were counted using a Zeiss binocular microscope at 10 – 100x magnifications. Root tissue, excluding the hypocotyl were then weighed and stored immediately at - 20 °C. Roots were refrozen in LN_2 before grinding and examined by western blot analysis or root squashes according to the methods previously outlined in sections 2.2.20 and 2.2.24 respectively. Dot blot tests were performed for bioassays 4 and 5 by pipetting 10 µl root extract onto a nitrocellulose filter and air-dried for 30 minutes. Filters were then blocked, probed and developed according to section 2.2.20.3.

B) Galls developing on tomato roots were counted 8 days after virus inoculation and processed according to A) above.

5.2.2.6 Statistical analysis

Data for *N. benthamiana* bioassays 1, 2 and 3 were not combined due to the differences in design and treatment replication. To accommodate for the variation in number of treatment replicates and the blocking of each treatment in rows, gall data were tested individually using analysis of variance (ANOVA). Tomato bioassays 1, 2 and 3 were also analyzed individually using ANOVA as above.

Statistical analysis was performed on combined sets of gall data for *N. benthamiana* bioassays 4 to 6 and bioassay 7 to 10. Gall data were tested using ANOVA with a 'complete randomized block' blocking structure, composed of 10 blocks, each containing one replicate of each plant treatment. All gall counts were square root transformed to normalize data since variance among the treatments was not homogeneous. Data was then statistically analyzed by ANOVA using Genstat 5 (windows).

5.3 Results

5.3.1 Root knot nematode bioassays

N. benthamiana or *L. esculentum* plants were grown in glasshouse conditions for 27 days and inoculated with the experimental treatments. At 7-9 dpi roots were challenged with infective *Meloidogyne* spp J2 nematodes. Ten to 14 DAI (days after infestation and potentially root invasion), root tissue was harvested and the number of root galls counted (see Figure 5.1). Each series of bioassays are described separately according to the experimental design, nematode population or host plant.

5.3.2 *N. benthamiana* bioassays

5.3.2.1 Observations of root galling for bioassays 1 to 3

Statistical analysis by ANOVA indicated that galling by the mixed *Meloidogyne* spp population had been lowered on roots infected by TRV-GNA for the individual *N. benthamiana* bioassays, but only significantly for bioassays 1 and 2 (see Table 5.1). The mean number of galls for each treatment and bioassay are presented in Figure 5.2. For bioassay 1, the mean gall counts on TRV-GNA infected plants were 1.87 sqrt galls (5 galls) compared to 4.66 sqrt galls (23.6 galls) on virus-free (sed 0.619 (galls sed 5.98); $p<.001$). For bioassay 2, 4.11 sqrt galls (21.2 galls) were counted on TRV-GNA infected roots compared to 5.79 galls (35.2) on virus-free roots (sed 0.660; $p<.018$). Similarly for bioassay 3, 4.10 sqrt galls (18.9 galls) were counted on TRV-GNA inoculated plants compared to 4.88 sqrt galls (24.7 galls) for virus-free plants (sed 0.507; NS). For the transformed data, a comparison of mean galls counted on the above treatments indicates that GNA reduced the level of galling by 60 %, 29 % and 16 % for bioassays 1 to 3 respectively compared to virus-free roots.

In bioassays 1 and 3 the proportion of galling on plants infected with TRV-fsGNA were not different from the mean galling on the virus-free plants (see Table 5.1) (NS, ANOVA). However, for bioassay 2 (see Table 5.2), plants infected with TRV-fsGNA produced a significantly lower mass of roots (0.526 sqrt g (0.305 g)) compared to either virus-free or TRV-GNA infected plants (0.592 sqrt g (0.355 g) and 0.625 sqrt g (0.395 g)

Table 5.1 A) Summary of analysis of variance of gall counts for individual nematode bioassays 1, 2 and 3 blocking for treatment only. B) Summary of analysis of variance of gall counts for combined data for nematode bioassays 4 to 6 and 7 to 10, blocking for bioassay only .

A

	Bioassay 1				Bioassay 2				Bioassay 3			
Source of variation	d.f. (m.v)	m.s	v.r	F pr.	d.f. (m.v)	m.s	v.r	F pr.	d.f. (m.v)	m.s	v.r	F pr.
Treament	2	25.957	13.55	<.001	2	12.791	4.40	0.018	2	4.492	2.61	0.082
Residual	24 (3)	1.915			45 (1)	2.904			58	1.718		
Total	26 (3)				47 (1)				60			

B

	Mean number of galls							
	Bioassays 4 to 6				Bioassays 7 to 10			
Source of variation	d.f. (m.v)	m.s	v.r	F pr.	d.f. (m.v)	m.s	v.r	F pr.
Bioassay	2	6.7755	3.41		3	7.369	6.21	
Bioassay.Block	27	1.983	0.83		36	1.186	1.03	
Treatment	2	9.399	3.95	0.025	3	5.611	4.86	0.003
Residual	56 (2)	2.380			113 (4)	1.155		
Total	87 (2)				155 (4)			

Figure 5.2 Bioassays 1 to 3. Galling induced by *Meloidogyne* spp nematodes on *N. benthamiana* roots recorded after 10 days. Gall counts are expressed as square root of data. Untransformed data are shown as labels.

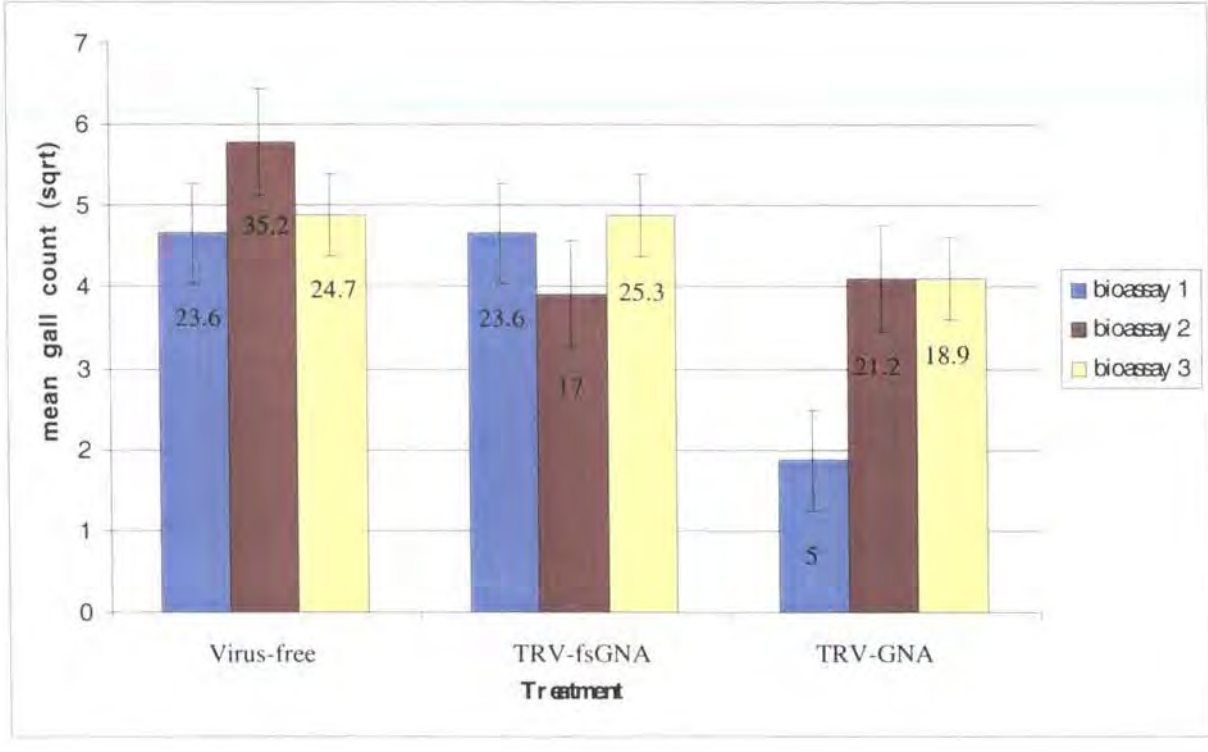
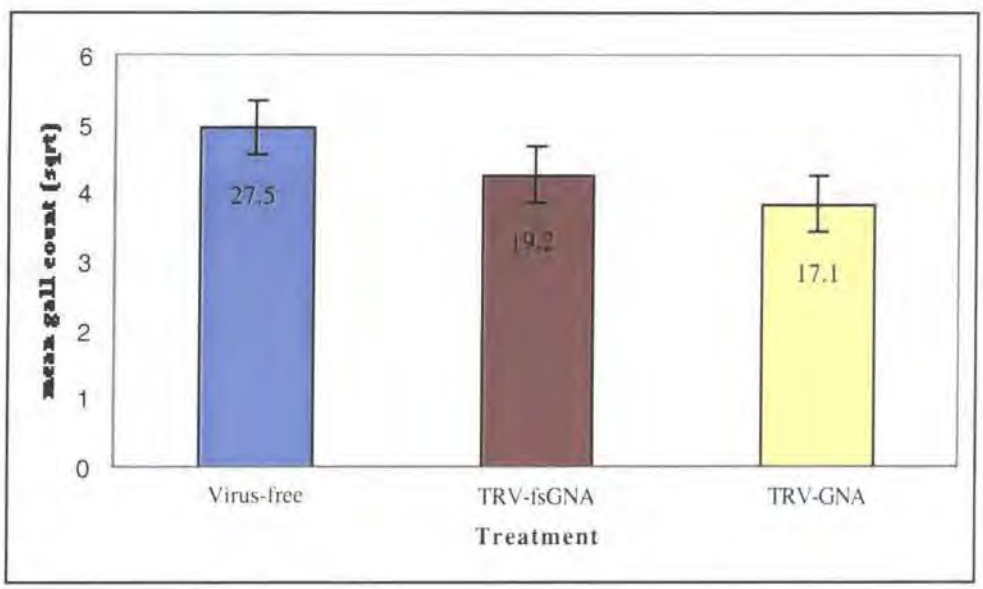
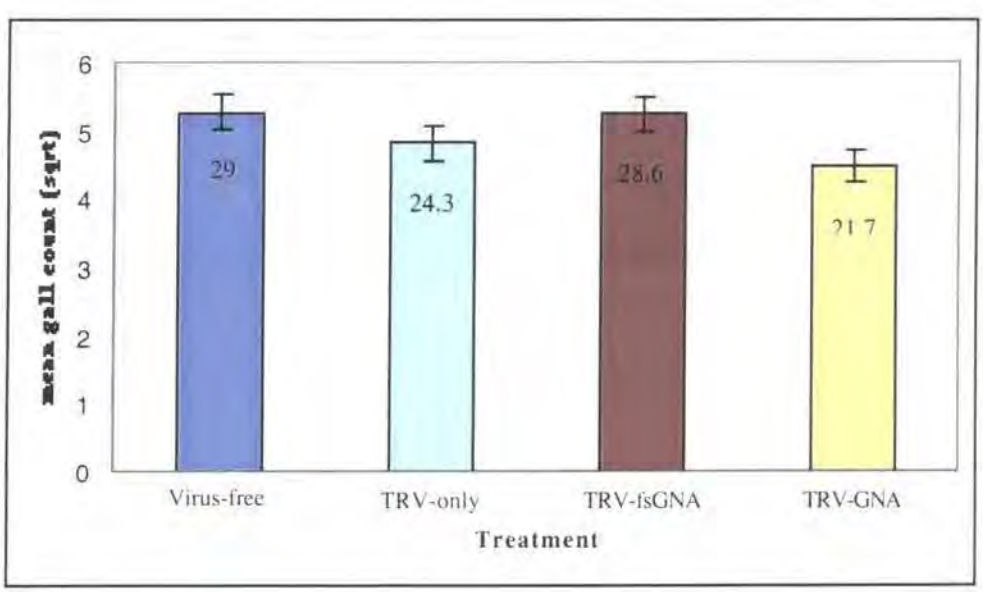


Figure 5.3 Mean number of galls (sqrt transformed) counted on virus-free *N. benthamiana* roots compared with roots infected with TRV-GNA or TRV-fsGNA. Panel A) is *Meloidogyne* spp data for bioassays 4 to 6. Panel B) is *M. javanica* Crete line 17 data for bioassays 7 to 10. Untransformed gall data is shown as data labels.

A



B



respectively) after analysis by ANOVA (p .045). Consequently, the number of galls counted on TRV-fsGNA infected roots (3.91 sqrt galls (17 galls) was significantly lower compared to virus-free (see above) and therefore probably the result of a negative effect from this treatment in this particular bioassay. Root weights measured for each treatment were not different for bioassays 1 or 3 (NS, ANOVA) (see Appendix I).

Table 5.2 Analysis of variance of fresh root weights for nematode bioassay 2 (Table I A, blocking for treatment). Mean root weights are shown in Table B (untransformed weights are included in brackets

A

	Bioassay 2			
Source of variation	d.f. (m.v)	m.s	v.r	F pr
Treatment	2	0.04916	3.31	0.045
Residual	45 (1)	0.01483		
Total	47 (1)			

B

	Treatment			
	virus-free	TRV-fsGNA	TRV-GNA	s.e.
SQRT mean root mass (untransformed)	0.592 (0.355)	0.526 (0.305)	0.625 (0.395)	$0.0545X^{min}$ $0.0472^{min-max}$ $0.0385X^{max}$
Rep	10	20	19	

5.3.2.2 Observations of root galling for bioassays 4 to 6

Statistical analyses of galling by *Meloidogyne* spp. are presented in Table 5.1 B) and indicate that significant differences in galling was related to treatment effects ($p<.025$) rather than differences between bioassays (NS, ANOVA). The combined gall data for *N. benthaminana* bioassays 4, 5 and 6 are presented in Figure 5.3 A and demonstrate that galling in roots infected by TRV-GNA (3.83 sqrt galls (17.1 galls)) and TRV-fsGNA (4.26 sqrt galls (19.2 galls)) were lower than galling on virus-free roots (4.94 sqrt galls

(27.5 galls)) (sed 0.398; p<.025). For the transformed data, a comparison of mean galls counted indicates that GNA and the truncated GNA protein decreased galling for this nematode population by 22.5 % and 14 % respectively when compared to virus-free roots. A comparison of fresh root weights indicated that although the least root mass was harvested from TRV-fsGNA roots, no differences were detected between each treatment (NS, ANOVA) (see Appendix I for results table).

5.3.2.3 Observations of root galling for bioassays 7 to 10

Statistical analyses of galling by *M. javanica* Crete line 17 are presented in Table 5.1 B) and demonstrates that differences in mean gall development was related to treatment effects (p<.003) rather than differences between bioassays (NS, ANOVA). The combined mean gall data for *N. benthaminana* bioassays 7 to 10 are presented in Figure 5.3 B) and demonstrate that galling was significantly reduced in roots infected by both TRV-GNA (4.487 sqrt galls (21.66 galls)) and TRV-ΔGFP 4.816 sqrt galls (24.31 galls) compared to roots infected by TRV-fsGNA (5.241 sqrt galls (28.61 galls)) and virus-free roots (5.273 sqrt galls (29.02 galls)) (sed 0.2403; p<.003). For the transformed data, root galling by *M. javanica* Crete line 17 was therefore reduced by the presence of GNA (21.6 %) and virus (8 %) when compared to galling on virus-free roots. In contrast to bioassays 4 to 6, galling on roots expressing the truncated GNA protein was not different to galling on virus-free roots (NS, ANOVA). Root weights were not analyzed for this series of bioassays as some root galls were large (see Figure 5.4) and thus would represent a large portion of the total root biomass (Scholte, 1990).

5.3.3 Tomato bioassays

5.3.3.1 Observations of root galling for bioassays 1 to 3

Analysis of gall data for tomato bioassay 1 indicated that galling on roots containing GNA were higher than for virus-free roots (sqrt 3.43 (15.2 galls) or roots expressing the truncated GNA protein (sqrt 4.49 galls (27.4 galls) (±0.963; p<.02). Root weights were also marginally higher for plants infected with TRV-GNA (0.2158 g) compared to virus-free (0.1614 g) and TRV-fsGNA infected plants (0.1506 g) (±0.0284; p<.037). The

observed increase in root weights for TRV-GNA infected roots may be related to gall mass (unmeasured in this experiment).

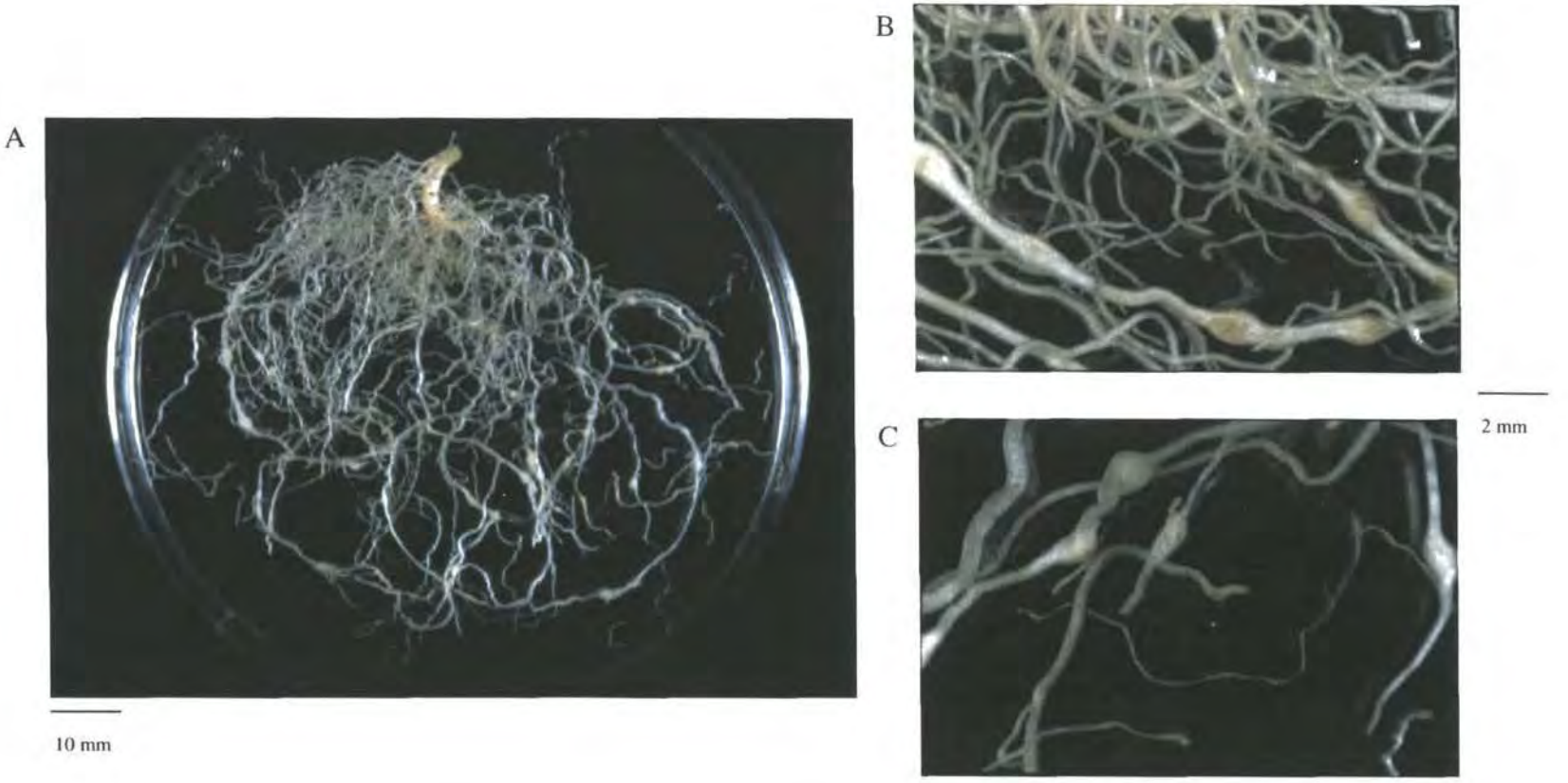
For tomato bioassays 2 and 3, root galling by the mixed *Meloidogyne* spp population was not significantly reduced on roots infected by TRV-GNA (26 galls and 48 galls respectively) compared to virus-free roots (25.7 galls and 55 galls respectively) (NS, ANOVA). Root weights (wet weights) from bioassay 2 were similar for each treatment (NS, ANOVA). Roots masses collected from TRV-GNA (0.363 g) infected plants in bioassay 3 were marginally smaller compared to virus-free (0.456 g) and TRV-fsGNA infected plants (0.442 g) ($p<.049$).

5.3.4 Detection of virus expressed proteins in roots infected by *Meloidogyne* nematodes

Attempts to quantify root extracts by spot tests (bioassays 4 and 5) were not successful as anti-GNA antibodies also bound to the virus-free and TRV-only controls. Analysis by western blotting (bioassays 4 to 10) produced blots with smeared banding which indicated that protein degradation had occurred. This was attributed to a long period of storage at –20 °C before analysis.

Four TRV-GNA infected root systems were harvested from bioassay 6 after 10 DAI and analyzed by tissue printing. The print in Figure 5.5 demonstrates variation in both the level of GNA was detected in the root tissue and gall size. Large discrete galls present on the roots are conspicuous as black, rounded, globular areas (D). Regions of adjacent galls were distinguishable as thickened humped areas of root with a chained appearance (C). Small galls were detected on roots containing the lowest levels of GNA (S).

Figure 5.4 Panel A Pertri-dish of *N. benthamiana* roots galls by the root knot nematode (*Meloidogyne* spp.) 14 days after infestation (DAI). Panels B and C show variation of gall development.



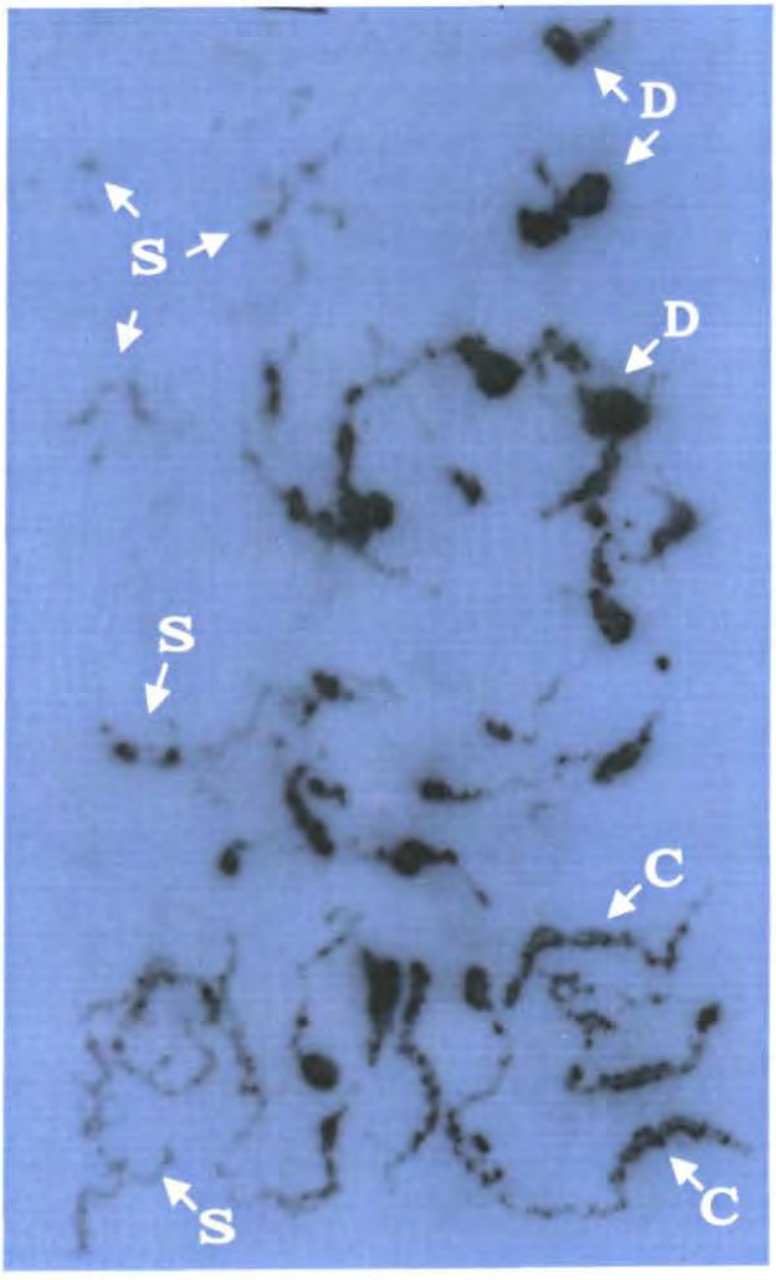


Figure 5.5 Tissue print of TRV-GNA infected roots showing localization of virally expressed GNA in galls induced by *M. javanica* after 10 days. Root gall are labelled as discrete (D), chains (C) or small (S). GNA was detected by GNA specific antibodies.

5.4 Discussion

The data from the current nematode bioassays demonstrate that plants expressing GNA in the root tissue are not resistant to infestation by J2 root knot nematodes.

5.4.1 Characteristics of virus expression

The nematode bioassay system is a combination of four main influences including the host plant, the nematode population, the virus vector and environmental conditions. The design of the bioassays was therefore standardized to limit variation between each experiment.

Firstly, *N. benthamiana* plants were propagated in sieved sand (500 – 1000 μm), moderate to severe galling by *M. javanica* is associated with coarse-textured soil (Barker *et al.*, 1985), on a small scale in 30ml pots. This also reduced the volume of substrate around the plant roots and limited the migration distance of the juvenile nematode (J2). Transferral of germinated seedlings that had an undeveloped root system ensured that root growth through the sand substrate would be normal and undamaged. Root knot nematodes are attracted to damaged roots (root exudate), which would interfere with trends resulting from the presence of virally expressed proteins.

Secondly, to limit variation in the nematode populations, specimens were collected from stock lines held at SCRI that had originated from single egg masses. Unfortunately, contamination of the *M. incognita* L27 population by *M. javanica* was detected (personal communication by Mark Phillips) and therefore nematodes used in bioassays 1 to 6 were designated as *Meloidogyne* spp.. Accurate identification of nematode species and race is important to enable correct recommendations for crop rotation and planting of resistant cultivars which are race specific (Hartman and Sasser, 1985). In terms of inocula viability, analyses of gall data indicated that the mean number of galls counted on virus-free roots varied between 23.6 and 35.2 for individual *N. benthamiana* bioassays 1 and 2 respectively and for the combined bioassay data galling varied between 28 galls (mixed population) and 29 galls (*M. javanica* Crete line 17). This suggested that approximately the same number of viable J2 per aliquot survived storage at 4°C and were being inoculated on to each plant. A major advantage of the virus-based system was that sterile

J2 were not required. In comparison J2 inoculated to hairy root cultures require surface sterilization or have to be reared in sterile conditions.

Thirdly, time-course and root squash experiments undertaken in Chapter 4, suggested that GNA expression was uniformly spread in root tissue after 9 dpi and present in root extracts from 6 to 24 dpi, a period that would accommodate the nematode bioassays (10 to 14 days). In contrast hairy root tissue generated from Agrobacterium transformations require sub-culture and are only available after approximately 3 months. GNA was also detected in roots after transferral to tropical conditions.

Fourthly, J2 nematodes would be exposed to virally expressed GNA which is a biochemically uniform substance compared to studies that apply drenches prepared from tissue extracts or oils (Gupta and Sharma, 1991). Fifthly, plants were evenly spaced in an enclosed propagator (30 cm x 48 cm) and under controlled glasshouse lighting. Differences in environmental conditions in this small area were expected to be minimal compared to a large-scale glasshouse study.

Lastly, for the preliminary *N. benthamiana* and *L. esculentum* bioassays (1 to 3) the number of replicates used for the virus were prepared in excess as the success of the virus expression system as its compatibility with nematodes was unknown. It was also assumed that infection of tomato by the TRV based expression vector would be similar to *N. benthamiana,* as tomato is regarded as a host for this virus, but ideally root squash and time course experiments should have been carried out for this plant. Ten replicates for each treatment were included in the bioassay design for experiments 4 to 10. Bioassays were repeated to accommodate for differences in J2 viability (see above) and plants. Data for each series of bioassays (4 to 6 and 7 to 10) were combined, as experiments were not significantly different. Low variance and consistent standard errors from the combined bioassays (although the variance for bioassay 1 was high) demonstrate that the virus expression system was successful and capable of producing a robust account of galling for each nematode line.

5.4.2 The effect of virally expressed proteins on nematode galling

Preliminary experiments in Chapter 3 and 4 indicated that a high level of GFP and GNA accumulated in *N. benthamiana* roots. Bioassays using the *Tobacco rattle virus* based expression vector was therefore an ideal opportunity to evaluate both the transient virus expression system and the performance of GNA as a biological nematicide. The root knot nematode was selected as the pest for this study because it is an important agricultural pest that will establish and complete its life cycle in *N. benthamiana*. Studies of lectin exposure to root knot nematode in the published literature were generally related to analysis of the surface coat, amphidial excretions and identification of populations. Therefore results from this current study was compared to methods designed to screen for nematode resistant cultivars.

This study reports on the affects of virally expressed proteins, specifically GNA, on galling by the root knot nematode. The test scores were based on the detection of gall in *N. benthamiana* roots at 10 to 14 days after infestation which were a visible index of feeding sites initiated by the female nematode at an early stage in development (see Figure 5.4).

5.4.2.1 A significant reduction in root galling is not indicative of nematode resistance

An analysis of gall data was undertaken to quantify GNA's control efficiency. The results from this study (bioassays 4 to 6 and bioassays 7 to 10) indicate that galling by both *Meloidogyne* populations were significantly reduced in TRV-GNA infected roots (3.83 sqrt galls and 4.487 sqrt galls respectively) compared to the virus-free treatment (4.94 sqrt galls, sed 0.398; $p<.025$ and 5.273 sqrt galls, sed 0.2403; $p<.003$ respectively) (see Figures 5.3 and Table 5.1). Data from bioassays 1 to 3 will not be discussed, as it is unclear whether the decrease in gall detected over the experimental period was related to the levels of virally expressed protein or corresponded to the unmeasured changes in dynamics of the mixed *Meloidogyne* spp. population. However, although a significant reduction in galling was detected for bioassays 4 to 10, it does not necessarily mean that GNA confers a significant level of nematode resistance since the extent of control achieved by GNA (analyses of transformed data) was only 22%. The terms 'resistant' and

'susceptible' are arbitrary and dependent on the plant host, environmental conditions (soil type, humidity, or temperature) and parasite pathotype (Geibel, 1982). Previous studies by Schmitt and Shannon (1992) describe a scheme that compares the female index to susceptible hosts and where the host response is studied separately from race. In this study if the female index % was > 60 % the soybean was susceptible, 31-60% moderately susceptible, 10 – 30% moderately resistant and 0-9% resistant soybean. The data from these current bioassays therefore indicates that although the presence of GNA reduces the susceptibility of roots to the root knot nematode, the actual level of control attained would not be considered as protective. However, the overall result does suggest that the response of the *Meloidogyne* genus to GNA was distinct and consistent. Therefore the effect of similar proteins might be predicted and quantified using the TRV virus expression system.

5.4.2.2 *M. incognita* was more susceptible to the truncated GNA protein than *M. javanica*

In contrast, to the effects of GNA mentioned above, variability in response to truncated GNA was detected for each root-knot nematode population. Galling in roots expressing TRV-fsGNA were 4.26 sqrt galls (bioassays 4 to 6) and 5.241 sqrt galls (bioassays 7 to 10) compared to the virus-free treatments (4.94 sqrt galls and 5.273 sqrt galls respectively). This result indicated that the two nematode lines had different parasitic capabilities with respect to their galling abilities in the presence of truncated GNA with the mixed *Meloidogyne* population being more susceptible to its presence. Variability within root knot nematodes species and races has been previously documented during the characterization of host plant status (Hartman and Sasser, 1985) and testing of resistant cultivars (Roberts 1998). This finding therefore emphasizes firstly, that the putative GNA protein is distinguishable from truncated GNA. Secondly, that the observed effects of an anti-feedant protein may be dependent on the differences in genetic background of the nematode, like an alteration or absence of surface glycans, which would be an important consideration for the selection of resistant pathotypes within the nematode community. Thirdly, consequently it was speculated, that if the level of galling observed for bioassays 4 to 6 is representative of a mixed population. Then presumably the reduced level of galling

reflects the *M. incognita* present in the inoculum (approximately 30 % if calculated from untransformed gall data) since no similar effects were detected for *M. javanica*.

Analyses of bioassays 7 to 10 suggest that plants infected by virus were marginally less attractive hosts to *M. javanica* than roots expressing the truncated GNA protein. But, the interaction of TRV particles on root invasion and root gall is difficult to interpret, as affects from each of the virally expressed foreign proteins were distinct despite the presence of the virus.

5.4.3 Plant resistance to nematode invasion

Mechanisms of plant resistance to nematodes can be described in two stages. The first pathway of nematode resistance is 'passive (pre-infectional) resistance' which is dependent on: 1) the host plant's ability to prevent invasion by either reducing the population of J2 around the root tissue (Geibel, 1982) or by interfering with host location. Previous studies of lectins and nematodes have shown that lectins bind to amphidial secretions and to the surface coat; 2) prevention of oncogenesis by either altering nematode sexuality due to poor tissue quality (Triantaphyllou 1973). Host suitability influences the sexual fate of *M. incognita* males (Sijmons *et al.,* 1994). Treatment of plants with maleic anhydrazide also increases the male to female ratio of *M. javanica* and *M. incognita* (Davide and Triantaphyllou, 1968).

The second pathway of nematode resistance is active (post-infectional) resistance which occurs after actual contact with the nematode and is based on a tissue hypersensitivity response (Geibel, 1982). Developed galls but an absence of nematodes have been reported by Ogallo *et al.*, (1997), it was speculated that after root penetration and initiation of gall that nematodes were killed by induced defence mechanisms.

Evidence from this study indicates that typically 78 % of the J2 locate, penetrate and initiate feeding sites in roots expressing GNA. Consequently, 22% of the infective J2 population are susceptible to the presence of this lectin and do not initiate gall. Reasons for the prevention of infestation by susceptible J2 is discussed. Although an explanation of GNA's affects is limited as information collected in this study was based on one parameter, which will not allow accurate evaluation of the complex interaction between an

animal and pesticidal protein. Data on passive resistance (sex reversal and nematode development) could be measured in future studies by incorporating image analysis *in situ* hybridization and microscopy to investigate this relationship further.

A primary assumption made by this technology was that the nematode would either ingest or be directly exposed to the pesticidal protein during development resulting in a toxic or inhibitory effect. In the case of root-knot nematodes migration through the root tissue is intercellular which results in minimal mechanical damage to the root cells (Sijmons *et al.,* 1994) and consequently a reduced level of exposure to GNA. Post-infectional resistance by the host plant to this pathogen is therefore potentially low. Necrosis is often associated with root-galling and can be rated similarly to galling from 0-100% (Barker, 1985). However, necrotic galls were not apparent within the root systems (see Figure 5.4 A) and the discoloured gall tissue shown in Figure 5.4 B was thought to be the body of the expanding globose female as the cortex cells separate rather than a host response, although this observation was not investigated further.

Nematodes are known to down-regulate genes in the cells within the feeding site (Goddijn *et al.,* 1993) which may also block enzymes required for defence mechanisms in the parasitized cells (Sijmons *et al.,* 1994). Tissue prints from this study (Figure 5.5) indicated that virally expressed GNA accumulated at the nematode feeding site, particularly in large galls, although the precise cellular location of GNA was not identified in this study. Further, this result suggested targeting expression of GNA at the nematode feeding site (NFS) would not guarantee toxic effects. This observation conflicts with Goddijn *et al.,* (1993) who speculated that targeting expression of nematicidal proteins inside the nematode feeding site would ensure a level of resistance that would be of commercial value.

Further studies would be recommended that would determine the contents of the different sized galls (distinguishing numbers of juveniles and adults). For example, by staining the root tisue with 0.05% acid fuchsin in lactophenol and cleared in lactophenol (Barker, 1985)

Feeding sites initiated by root-knot nematodes are formed without cell wall dissolution (Sijmons *et al.,* 1994) which suggests that detection of concentrated levels of GNA in the gall centre was related to increased permeability of the cell walls in the surrounding tissue and an active NFS. Further, this suggests that the GNA molecule was not excluded from the NFS by size limitations imposed by plasmodesmata and that cell to cell movement does occur. Detection by anti-GNA antibodies at the NFS demonstrates that the proteolytic enzymes secreted by a feeding nematode do not completely degrade GNA.

However, while the tissue prints confirm the presence of GNA in the gall tissue, the maximum molecular size of the anti-nematode protein is ultimately limited by the pathogens feeding tube (Atkinson *et al.,* 1992). Alternatively, the high concentrations of GNA in the large galls may be an artefact related to the squashing of a larger mass of tissue. Although the tissue prints suggest that a correlation between the presence of GNA and gall size may exist since large galls contain higher levels of GNA compared to small galls.

5.5 Conclusion

Analyses of gall count data obtained from this present series of nematode bioassays demonstrate that the virus expression system could be used to rapidly examine the effects of proteins, like GNA, on *Meloidogyne* spp. Although the relationships between the host plant, amount of gene product and the population of nematodes are complex.

1) Effects of GNA were detectable as differences in gall after 10 to 14 days.

2) High levels of GNA detected in large root galls were not toxic to this nematode and did not interfere with feeding site development. GNA present in *N. benthamiana* roots would not confer resistance to colonization by the root knot nematodes (*M. javanica* Crete line 17 or mixed population of *M. incognita* L27 and *M. javanica*).

3) Variability in root knot lines was detectable as differences in the galling response to the TRV-fsGNA treatment.

4) Plant material was available for testing after 8 dpi compared with 3 months for Agrobacterium-based studies. The design of the nematode bioassay could be adapted for more extensive physiological studies.

5) Virus-based bioassays do not require sterile J2 nematodes.

6) Nematode exposure to virally expressed proteins may by-pass the problems associated with reduced gene expression at the nematode feeding site.

In conclusion, the virus-expression bioassay could be used as a preliminary screen for the selection and investigation of nematicidal proteins. To determine whether lower levels of GNA expression would constitute higher levels of resistance to root-knot nematode invasion it was proposed that further studies using a virus expression vector based in *Pepper Ringspot Virus* would be a useful comparison.

Chapter 6

Effect of snowdrop lectin on nymph development and fecundity of the Glasshouse-potato aphid, *Aulacorthum solani*

6.1 Introduction

The anti-metabolic effects of GNA have been demonstrated in many homopteran studies. For example, *M. persicae* (Sauvion *et al.*, 1996), *A. solani* (Down *et al* .,1996), *A. pisum* (Rahbé *et al.*, 1995; Sauvion *et al.*, 1995), *N. lugens* (Powell *et al.*, 1998; Foissac *et al.*, 2000). Data from these latter and other studies indicate that the insect-lectin relationship is highly complex and dependent on many factors including the multi-mechanistic effects of the lectin, insect species, ecology and physiological effects pre- and post-digestion (see section 1.6.2). The major trends in toxicity include; the high susceptibility of oligophagous aphids which feed on a narrow host-range (Rahbé *et al.*, 1995; Sauvion *et al.*, 1995), reduced fecundity related to exposure to GNA throughout nymph development (Down *et al.*, 1996) and anti-feedant effects measured as reduced honeydew excretion (Powell *et al.*, 1995a). Although, after an initial aversion recovery has been recorded for short-term exposure to lectins (Gatehouse *et al.*, 1996; Powell *et al.*, 1998).

Vision and olfaction are important factors involved in the finding and recognition of a host plant. Although, it is possible to re-condition some insects to feed on alternative plants without affecting feeding behaviour (for example, *A. solani* in this study). Feeding activity of aphids (phloem ingestion) is usually initiated after explorative stylet probing of leaf tissue and an assessment of stimuli which are a balance between chemical factors relating to the taxonomy of the plant and nutritional factors that provides information on the physiological condition of the plant (Chapman 1978). The fact that homoptera and other insect species initiate feeding on phloem or tissue containing lectin suggests that visual cues are unaltered and although subsequent feeding may not be optimal, if the insect's movements are inhibited, then feeding may continue despite the presence of lectins. A major advantage of *in planta* bioassays is therefore an accumulation of anti-feedant proteins in plant tissue without altering either visual, olfactory cues or potentially the feeding behaviour of the candidate insect.

6.1.2 Aim

The main objective of the following study was to assess the potential of the TRV virus expression system as a rapid method of screening anti-feedant proteins, *in planta,* against aphid pests. To promote the 'rapid' aspect of these bioassays the experiment design focused on several specific criteria in the aphid life history that could be used to establish a response to the virus treatments.

6.2 Materials and Methods

6.2.1 Insects

A Scottish *Aulacorthum solani* (Kaltenbach) clone was obtained from Dr T Woodford, Scottish Crop Research Institute. The aphid clone was then continuously reared on 4 to 8 week old *N. benthamiana* plants under environmentally controlled conditions (20 °C with 16 : 8 hours (light : dark). The axial and abaxial leaves surfaces of all plants were washed with 0.05% Teepol before the plants were transferred to the perspex rearing cages. The snowdrop is not a host plant for *A. solani* clone. The clone used in this research had no prior exposure to the snowdrop lectin.

6.2.2 Whole plant aphid bioassays

6.2.2.1 Plant inoculation

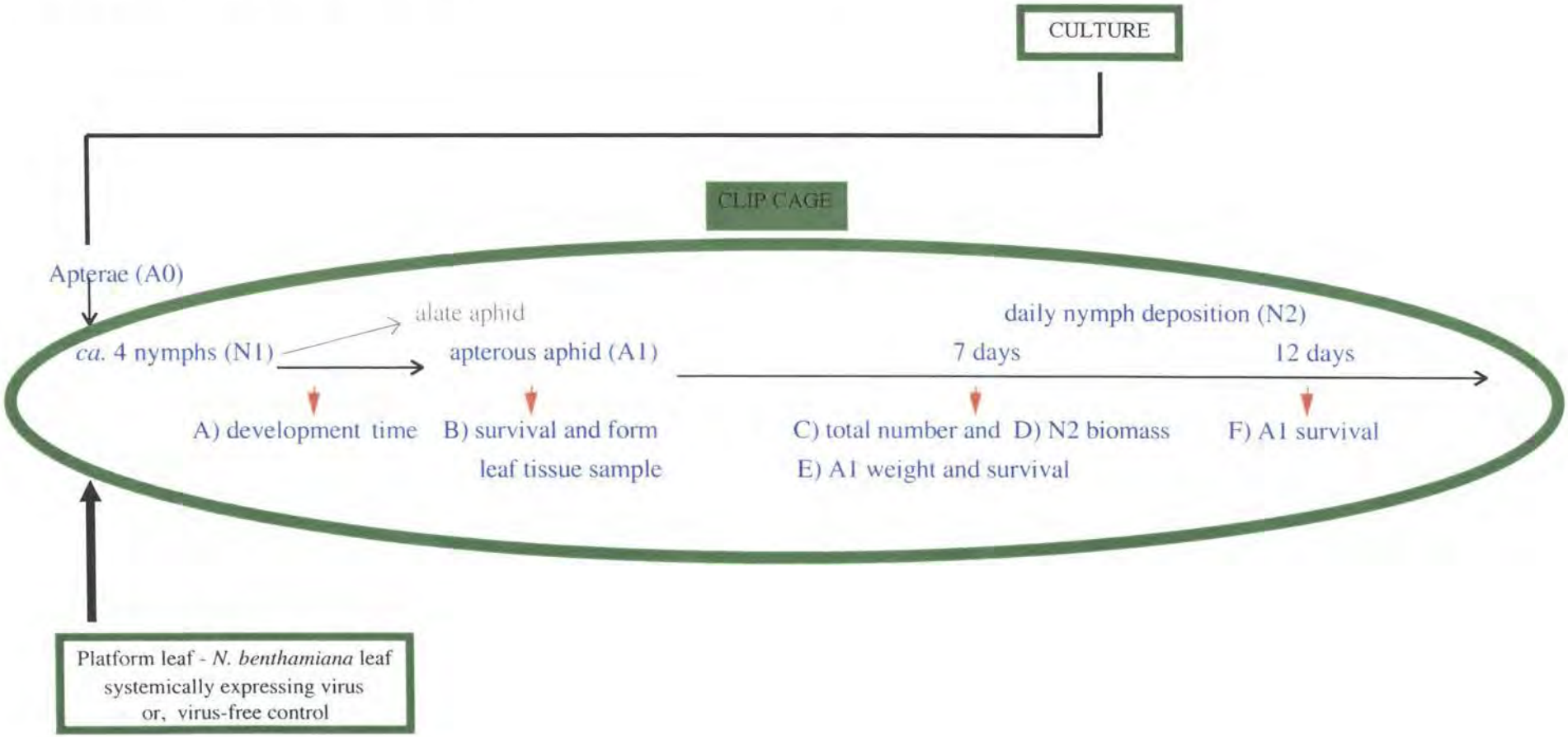
1) The standard virus inoculation protocol described in section 2.19 was followed for this study. For each bioassay, ten *N. benthamiana* plants were inoculated at 27 days old with one of the following treatments; TRV-GNA, TRV-fsGNA, TRV-ΔGFP and water only (mock-inoculated control). The progress of the virus infection was gauged by assessing systemic GFP expression from TRV-GFP infected plants that were inoculated at the same time. Plants were prepared for the bioassay at 6 to 7 dpi, when GFP was expressed uniformly across the platform leaf (test leaf).

2) Prior to the bioassay, the systemically-infected platform leaves were washed with 0.05% Teepol, rinsed with water and then allowed to air dry. The treated plants were then placed in a randomized, complete block design consisting of ten blocks containing a replicate of each treatment. The bioassay was contained in trays treated with P.T.F.E (ICI) to prevent aphid escape. All aphid bioassays were carried out in a licensed glasshouse set at 20 ± 1 °C and 16 h light : 8 h dark.

6.2.2.2 Aphid inoculation

The experiment was designed to compare the effects of four plant treatments on the life cycle of the aphid *A. solani.* The present bioassay was based on the design used by

Figure 6.1 A schematic representation of the rapid *in planta* Tobacco rattle virus expression bioassay protocol adapted from Gatehouse *et al.*, (1996), including the aphid responses monitored.



Gatehouse *et al.*, (1996), but applying a virus expression system as the method of GNA delivery rather than using transgenic plants.

To initiate the bioassay experiments mature apterous aphids (A0) were removed from a synchronised stock population using a moistened camel hair brush and starved for one hour. Individual A0 aphids were placed into a clip-cage positioned onto the abaxial surface of the platform leaf and monitored over 2-3 days until four first generation nymphs were produced; a varied time period was allowed to compensate for the high number of alatae developing from this culture, which was related to exposure to a sub-optimum light regime. The first day of nymph production (N1) was noted as day 0. In cases of premature A0 mortality, re-inoculations were carried out up to 2 days after initial infection but only when replacement aphids were available from an identical treatment source. The nymphs were then left undisturbed for 10 days and monitored daily until maturity (A1) and nymph production.

6.2.2.3 Assessment of aphid growth and development

A series of observations were recorded for two generations of *A. solani* aphids and were interpreted as a measure of aphid performance on four leaf treatments (virus-free, TRV-ΔGFP, TRV-GNA, TRV-fsGNA) for four consecutive bioassays (1, 2, 3 and 4). The assessment scheme is illustrated in Figure 6.1. The following data were collected:

A) The development time for all individual first generation of aphids was recorded as the period from birth to the first day of nymph production (second generation, N2), which for this study was used to indicate adulthood (A1).

B) The morphological form of all surviving adult A1 aphids was also recorded for each replicate and scored as: 'no survivors' (0); 'apterae only' (1); 'alatae only' (2) or 'both alatae and apterae' (3).

The parent A1 aphids (apterae) and deposited second-generation nymphs (N2) were retained. All immature aphids and alatae were removed from the clip-cage.

C) The cumulative daily reproduction of the caged A1 aphids was then recorded, at approximately the same time each day.

D) After a period of seven days the nymphs were collectively weighed and survival of the A1 aphid was assessed.

E) For bioassays 1 and 2 the surviving A1 aphids were weighed after 7 days.

F) For bioassays 3 and 4, the numbers of newly deposited nymphs were recorded for a further 5 days and removed daily. The A1 aphids were left undisturbed.

6.2.2.4 Leaf tissue samples

Two tissue samples were removed from the platform leaf per experiment using the lid of a 1.5 ml microfuge tube. The first leaf disc sample was removed at the onset of nymph production from the basal region of each leaf, opposite to the clip cage. Leaves were then re-sampled on the final day of the bioassay. All tissue samples were stored at -20 °C before examination by western blot analysis. Expression of GNA in the test leaf was then determined by densitrometric analysis of the western blotting membrane using Bio image® intelligent Quantifier™ Version 2.5.0 software (1994 - 1997) B.I. Systems Corporation.

6.2.2.5 Statistical analyses

Each of the *A. solani* responses was analyzed as follows: Treatment effects on the A1 aphid development time were tested using analysis of variance with a 'complete randomized block' blocking structure. A Pearson's chi square test was used to analyze differences in survival and morphology of the A1 aphid. The number of adult A1 aphids in each bioassay varied depending on the number of first generation nymphs that survived and developed into an apterous adult. To accommodate for this variation, the survival of individual A1 aphids, on consecutive days, during nymph production was analyzed using a binomial distribution, which tested the survival of individual aphids against the total number of aphids present at the onset of nymph production. A linear regression model was used to analyze the treatment effects on: a) the A1 aphid weight after 7 days nymph production; b) the total number of nymphs; c) combined nymph biomass; d) mean aphid weight after 7 days and e) mean aphid weight after 12 days. These data sets were normally distributed and therefore were not transformed before analysis. The linear regression

model was used rather than ANOVA because the latter approach estimates missing values rather than excluding them, and such estimations may distort the analysis. Statistical analysis for each aphid response was performed on a combined set of data (bioassays 1 - 4). The Genstat 5 (windows) statistical package was used for all analyses.

6.3 Results

6.3.1 Virus expression bioassay

Young *N. benthamiana* plants were grown in a temperature controlled glasshouse and inoculated with the experimental treatments seven days later. Plants were then exposed to *A. solani* aphids. The effects of TRV GNA proteins on aphid growth and development were then assessed.

Sections A to F refer to the stages of aphid growth and development assessed during this study (see Figure 6.1).

6.3.1.1 Observations of the first generation of aphids

A) Development times of nymphs to the fecund adult stage (A1) reared from birth on maturing leaves containing the virus only (12.0 days), TRV expressed GNA (11.9 days) or the TRV-fsGNA infected leaves (11.6 days) were not different from those developing on virus-free leaves (11.8 days; sed 0.26) (NS, ANOVA). Consequently, the onset of nymph production by these A1 aphids was not affected by the different treatments.

B) Aphid polymorphism and aphid survival over this developmental period was also unaffected on leaves containing virus or virus-expressed lectin. Analyses by Pearson's Chi square test indicated that at 9 d.f. no relation existed between each of the observed counts (no survivors; apterae only; alatae only or mix of alatae and apterae) and treatment.

The proportion of the A1 aphids surviving during the first 7 days of nymph production was not significantly different after analysis by binomial distribution. However, significant differences were detected for bioassays 3 and 4 for aphid survival at day 8 ($p < .01$), day 9 ($p < .05$), day 10 ($p < .05$) and day 11($p < .01$). Although analysis indicated that the variation was related to differences between bioassays rather than to treatment, and probably resulted from high external temperatures which raised the glasshouse temperature to 28 °C, resulting in high mortality rates during the latter part of bioassay 4.

Table 6.1 I) Linear Regression analysis summary of the variates total number of nymphs, total weight of nymphs and mean weight recorded after 7 days for the fitted terms: Constant + bioassay + treatment.

I

		Total number of nymphs			Total nymph weight (mg)			Mean nymph weight (mg)		
Source of variation	d.f.	m.s	v.r.	F pr	m.s	v.r.	F pr	m.s ($\times 10^{-5}$)	v.r.	F pr
Bioassay	3	153.2	2.94	0.038	0.0233	4.58	0.005	2.16	3.95	0.011
Treatment	3	62.20	1.19	0.318	0.0176	3.46	0.021	2.42	4.42	0.006
Bioassay.treatment	9	42.90	0.82	0.597	0.0042	0.82	0.603	0.275	0.50	0.868
Residual	75	52.09			0.0051			0.547		
Total	90	54.88			0.0060			0.636		

Table 6.1 Continued II) The predicted total number of nymphs, total nymph weight, mean nymphal weight and standard error predicted for each bioassay and treatment.

II

	Total number of nymphs	s.e ±	Total weight of nymphs (mg)	s.e ±	Mean weight (mg)	s.e ±
Bioassay						
1	20.3	1.68	4.98	0.526	0.255	0.0172
2	23.3	1.40	6.78	0.439	0.303	0.0144
3	24.5	1.38	6.00	0.431	0.242	0.0141
4	18.1	1.85	4.27	0.579	0.236	0.0190
Treatment						
Virus-free	24.0	1.37	6.77	0.428	0.29	0.0140
TRV-fsGNA	20.6	1.75	4.61	0.548	0.212	0.0180
TRV-GNA	22.3	1.55	5.41	0.485	0.245	0.0159
TRV-ΔGFP	20.7	1.57	5.54	0.492	0.276	0.0161

C) Aphid fecundity data (total number of nymphs produced) recorded on day 7 was not affected by the presence of virus (20.7 nymphs, ±1.57), virus expressed GNA (22.3 nymphs, ±1.55) or TRV-fsGNA infected leaves (20.6 nymphs, ±1.55) when compared to virus-free leaves (24.0 nymphs, ±1.37) by linear regression analysis (NS) (see Table 6.1). The results demonstrate that the rate of population increase for each treatment was similar for this period. Differences in aphid fecundity were detected between each of the bioassays ($p < .038$) (see Table 6.1). For example, the total numbers of nymphs were highest in bioassay 3 and 2 (24.5 and 23.3 nymphs respectively), and the least in bioassay 4 (18.1 nymphs). However, as treatment differences were not detected this result suggested that the differences originated from an external source, for example, the host plant population or an unmeasured environmental factor.

6.3.1.2 Observations for the second generation of nymphs

D) Nymph (N2) growth and development was assessed after a 7-day bioassay period. Initial analysis of the mean nymph weights (total nymph biomass of all nymphs/total number of nymphs at 7 days) indicated that there was a significant treatment effect on nymph development (p 0.006). The mean weights for each treatment are presented in Table 6.1 and demonstrate that nymphs ingesting phloem from leaves expressing either GNA (0.246 mg ±0.0159; $p < .01$) or the truncated fsGNA protein (0.212 mg ±0.018; $p < .001$) were significantly smaller than those feeding on virus-free phloem (0.290 mg ±0.014). In contrast, the presence of the TRV virus alone did not affect the growth of the nymphs (0.275 mg ±0.0161; NS). Analysis also indicated that the variation of the mean weights for each treatment was smallest between nymphs feeding on virus-free leaves and greatest between nymphs exposed to the truncated GNA protein.

A comparison of the mean nymph weights indicate that the weight gain by nymphs feeding on virus-free phloem was only 4.8 % greater than those feeding on phloem containing the unmodified virus, whereas nymphs exposed to GNA and fsGNA were smaller by 15.2 % and 26.6 % respectively. The results of the statistical analysis are presented in Table 6.1 and indicate that while significant variation existed between the individual bioassays ($p < .011$), the majority of the variation originated from bioassay 2

which consistently yielded larger nymphs (0.303 mg ±0.144). However, the absence of an interaction between the bioassays and treatments confirmed that the overall pattern observed in each treatment was consistent between each individual bioassay and thus indicated that a treatment effect was detectable in each individual bioassay.

Regression analysis of total nymph weight data on day 7 indicated that significant differences existed between both the treatments ($p < .021$) and the individual bioassays ($p < .005$). The results of the statistical analysis are presented in Table 6.1 and show that the trend observed for the total weights is similar to that observed for the mean nymph weights. Comparison of the total nymph weights indicated that the total weight gain by nymphs feeding on virus-free phloem (6.77 mg ±0.428) was 18 % greater than those feeding on phloem containing unmodified virus (5.54 mg ±0.492). In contrast, nymphs exposed to GNA (5.41mg ±0.485) and the truncated GNA protein (4.61 mg ±0.548) were smaller by 20 % and 32 % respectively. The absence of an interaction between the bioassays and treatments indicated that this treatment effect was detectable in each individual bioassay. However, analysis of aphid fecundity (total number of nymphs at 7 days) in section B revealed that all variation for this parameter originated from differences between individual bioassays which suggests that some of the variation observed for total nymph weight may have a maternal origin.

6.3.1.3 Observations for the adult aphids

E) The recorded weights of the A1 aphids was measured after 7 days of nymph production and subjected to linear regression analysis. The mean weight of the A1 aphids feeding on leaves containing the virus (1.42 mg), GNA (1.32 mg) or the frame-shift GNA (1.47 mg) were not different to those feeding on virus-free leaves (1.52 mg; sed 2.58×10^{-4}; NS) (see Table 6.2).

F) A1 aphid weights after a 12 day period were only recorded for bioassay 3 data, as premature mortality was observed for bioassay 4 (this correlated to extreme glasshouse temperatures). The mean weight of the A1 aphids feeding on leaves containing the virus (1.53 mg), GNA (1.7 mg) or the frame-shift GNA (1.53 mg) were not different to those

feeding on virus-free leaves (1.8 mg; sed 2.42 x 10^{-4}; NS) (see Table 6.2). However, for this bioassay, the adult aphids developing on leaves containing GNA were marginally larger than aphids developing on either leaves infected with TRV-ΔGFP or TRV-fsGNA.

Table 6.2 Mean A1 aphid weights recorded after 7 days (bioassays 1 and 2) and 12 days (bioassay 3 only) of nymph production

	Mean adult aphid weights (mg)			
Treatment	7 days (n=80)	s.e	12 days (n=40)	s.e
Virus-free	1.52	±0.069	1.80	±0.108
TRV-fsGNA	1.47	±0.084	1.53	±0.140
TRV-GNA	1.32	±0.077	1.70	±0.171
TRV-ΔGFP	1.42	±0.111	1.53	±0.091

The complete statistical output for each aphid response is presented in Appendix II.

6.3.1.4 Screening of treated plants for GNA expression

Leaf extracts of study leaves infected by TRV-GNA were collected from the bioassays after 7 days and analyzed by western blotting. The blots shown in Figure 6.2 indicate that similar levels of virally expressed GNA detected in the platform leaf (study leaf) are detected in an individual bioassay, but that variation exists between each of the four bioassays. Tissue extracts for bioassay 4 collected at day 7 (Blot 4) and day 12 (Blot 5) (the last day of the bioassay equivalent to 30 to 33 days post virus infection) are presented in Figure 6.2 and indicate that the level of GNA detected in each platform leaf decreases over this period.

Quantification of the GNA signal at day 7 by densitometric imaging estimated that for 32 out of 40 samples the level of GNA varied between 3 ng to 130 ng per 14.4 mg leaf disc. However, the programme was unable to calculate 7 leaf samples from bioassay 3 and 1 sample from bioassay 2 due to auto-radiograph quality. Figure 6.3 presents a $\log_{10}$ plot of the calculated amounts of GNA (ng) versus the nymph weight data (μg). The data suggest that leaf tissue containing higher levels of accumulated GNA inhibit nymph development. However, nymph mortality was detected for leaf samples containing both high and low levels of GNA.

Figure 6.2 Western blots of TRV-GNA infected leaf tissue sampled on day 7 of the aphid bioassay (sample A) for bioassays 1 to 4 (Blots 1 to 4) and day 12 (sample B) for bioassay 4 (Blot 5). Lanes are loaded with equal volumes of tissue extract; lane 1, 20 ng of GNA protein standard, lane 2-11 contains infected plants 1 to 10. Blots were probed with GNA-specific antibodies.

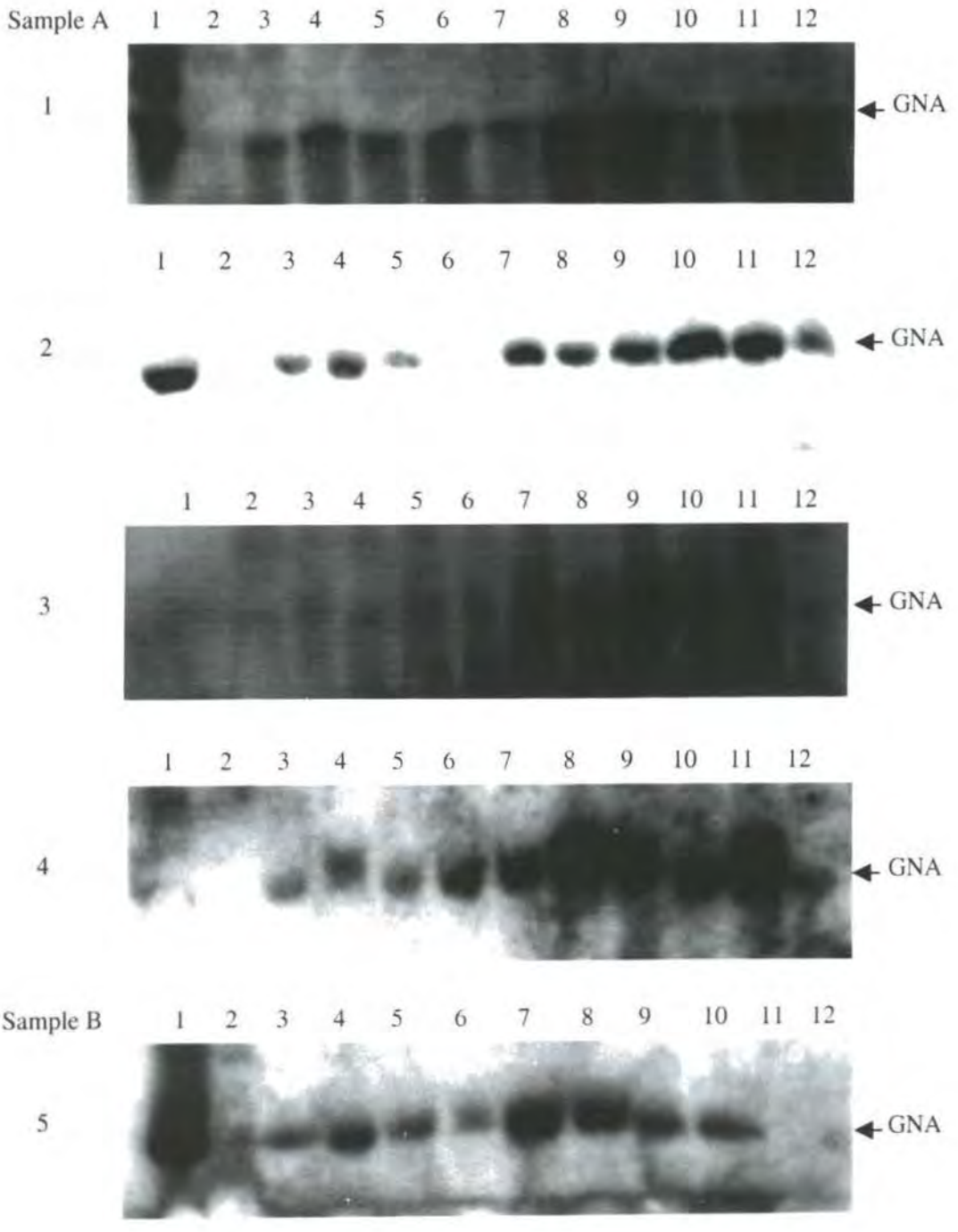
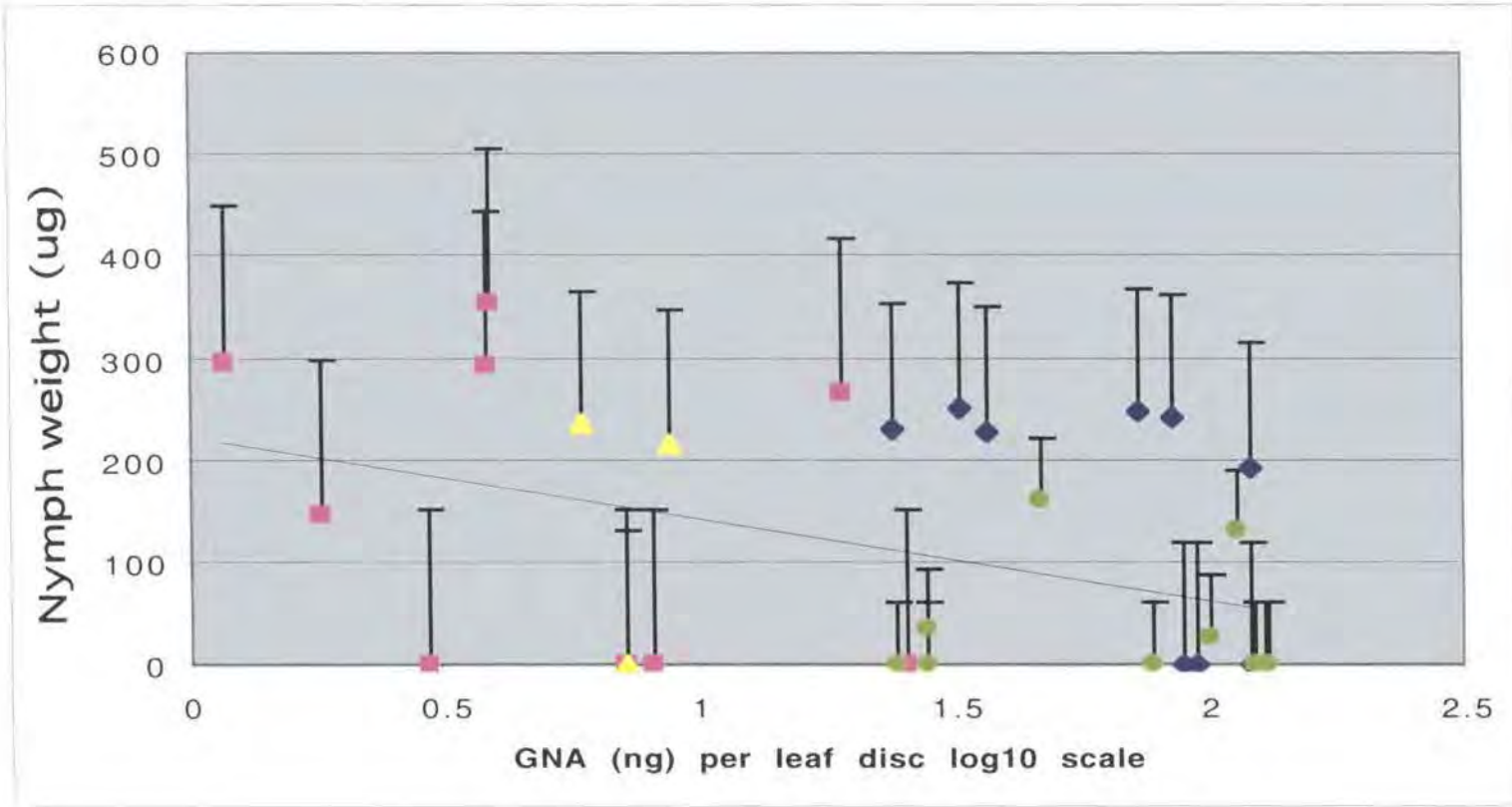


Figure 6.3 Trend of antibiosis of individual *A solani* nymphs measured as weights (µg) after feeding on leaves containing GNA (ng). Accumulated level of GNA was estimated by densitometric analyses. Bioassays are shown as 1, 2, 3 and 4



6.4 Discussion

The results obtained from the current aphid bioassays confirm the view that the relationship between the aphid and its environment is complex.

6.4.1 Characteristics of virus expression

The TRV virus expression system is a composite model in that it is a combination of four main influences; the host plant, the aphid colony, the virus vector itself and environmental conditions. Each bioassay therefore involves the interaction of three inherently variable biological systems. To limit the variation between each bioassay, the design was therefore standardized where possible.

Firstly, uniformly sized *N. benthamiana* plants were inoculated with second passage virus extract. A major advantage of the virus vector technology is that systemic GNA expression was detected after 6 dpi, which allowed a bioassay study to be initiated immediately. As previously shown in Chapter 3 and 4, the systemic viral infections were uniform so that it could be predicted which leaves would contain virus-expressed GNA. Thus, it was possible to attach the clip-cage at a specific position in the plant canopy and be confident that the virus would invade this leaf. The platform leaf was also compatible with *A. solani* feeding site preference; not all *N. benthamiana* leaves are equally acceptable to this aphid clone with preference given to the first leaves rather than the new leaves (personal observation). Differences in phloem quality and activity exist across the leaf lamina in relation to the source-sink activities of the leaf (Roberts 2000). The clip cage was therefore attached at a standard position near the leaf base.

Secondly, variability in the aphid population was reduced by selecting apterae from a clonal culture, although experiments were performed consecutively at different times of the year. Dense colonies of apterae are also the most prevalent polymorhic form isolated from developing plants in the growing season and therefore data from these bioassays may provide an advanced indication of effects in the field. Exclusion of the highly mobile alatae reduces variation related to differences in physiology (Guldemond *et al.*, 1998); although it is understood that information regarding the interaction of alatae and

transformed tissue would be important data for field trials. Aphid handling was also minimized for all experiments as this may affect *A. solani* survival (Down *et al.*, 1996).

Thirdly, the virally expressed protein was a biochemically uniform substance. Bioassay performance would, therefore, not be influenced by formulation as might occur when using purified protein in *in vitro* feeding experiments (Sauvion *et al.*, 1995). Previous studies suggested that durable protein expression levels were attained when using the virus vector system (described in Chapter 4), allowing comparisons between a series of plants.

Fourthly, the effects of external factors were minimized by maintaining the bioassays in a glasshouse set at 20 °C ± 1 °C and 16 hours light, although, a controlled environment cabinet would have provided the most stable conditions for the study. Ten replicates for each treatment (a total of 40 plants per experiment) were included in the bioassay design. This was regarded as an adequate number to accommodate for experimental variation and to provide meaningful statistical analysis. Repetition of the bioassay also accommodated for differences originating from the study of individual aphids rather than groups of aphids, for example, the differences between nymph generations (reported in Adams and van Emden 1972).

However, despite all the problems associated with the virus expression bioassay design, its success is reflected in the low variance that was observed for each of the measured response parameters.

6.4.2 Aphid performance

A. solani, was selected as the pest species for this study because it is an economic pest that will feed on *N. benthamiana*. Data was available from previous studies that identified the snowdrop lectin as being an anti-feedant towards several phloem-feeding pests including *A. solani*. Findings in these earlier studies were based on both *in vitro* (artificial diet) and *in planta* (transgenic plants) experiments and therefore provided an abundance of valuable information against which findings from the virus expression bioassay could be compared.

The results from this study document the effects of dietary GNA on *A. solani* performance and indicate that the aphid responses observed here were broadly consistent with the observations made for this species by Down *et al.,* (1996). In this study, statistical analysis indicated that all responses measured (development time, survival, adult weight, short-term fecundity after 7 days and adult survival during nymph production) for the first generation of aphids were unaffected by the presence of the TRV virus, GNA or the putative, truncated GNA protein expressed by the virus. No lag in nymph production was observed in the virus bioassay system, which was in direct agreement with the transgenic assay of Down *et al.,* (1996). Although a lag period was observed during the artificial diet assays (Down *et al.,* 1996) and for *M. persicae* feeding on transgenic lines expressing either BCH or WAI and GNA (Gatehouse *et al.,* 1996), which may suggest that differences in aphid response are related partly to the bioassay design and candidate insect. A significant reduction in the cumulative number of nymphs was observed by Down *et al.,* (1996) in the transgenic, glasshouse and dietary assays, together with a 10% decrease in first instar survival on artificial diet containing GNA. These effects were not observed in this current study of the first aphid generation. It is speculated that these earlier differences observed in aphid performance might be related to the particular host plant species, stage of plant development or aphid clone. In the current bioassay study, the *A. solani* were reared on 'developing' *N. benthamiana* plants resulting in a mean cumulative nymph count, after 7 days, of 24 nymphs (se 1.37) on virus-free leaves and 22 nymphs (se 1.55) on GNA-expressing leaves. However, when this aphid species was reared on 'mature' potato plants by Down *et al.,* (1996), similar counts were observed on control and GNA-expressing leaves only after approximately 17 days and 28 days respectively. This observation suggests that the intrinsic rate of population increase is dependent on one of the following factors; either host plant species, stage of plant development or aphid clone. Therefore, a direct comparison between the two sets of fecundity data might be misleading.

In an attempt to understand the variation of anti-feedant properties of the virally expressed GNA to *A. solani* observed in this study, the effects of plant development and host species were explored.

In terms of plant development, the leaf is a complex and continually changing substrate in that the quality of phloem varies over the growing season depending on the stage in plant development. Van Emden and Bashford (1971) previously demonstrated a positive correlation between soluble nitrogen and the intrinsic rate of aphid increase. To illustrate the importance of phloem quality, it was reported in Dixon (1998) that in natural aphid populations, larger, more fecund individuals developed in synchrony with bud burst, compared with aphids that were produced later in the growth season and are less fit. In terms of aphid performance and host plant species, differences were detected at the cultivar level for the Melon aphid, *Aphis gossypii,* when reared on chrysanthemum. But more importantly, this research reported an increase in reproductive performance correlating to foliar concentrations of soluble protein. Although high levels of applied nitrogen reduced insect performance by inducing salinity stress in the roots which consequently reduced soluble foliar protein (Bethke *et al.*, 1998).

The *N. benthamiana* plants for this bioassay study were inoculated with each virus treatment at approximately 27 days old (< 2 cm) when the leaf was actively growing and rapidly expanding. Phloem entering the young leaf (a nutrient sink) would therefore contain high concentrations of photosynthates (high quality phloem) which, according to Dixon (1998), would mean that feeding N1 nymphs (first generation) would only have to ingest small amounts of phloem to develop normally. In contrast, nymphs developing on a mature leaf, with low quality phloem (ie. the second N2 generation) would accordingly have to ingest larger volumes of phloem together with higher concentrations of the anti-feedant protein to attain normal growth. The results from this study, therefore, suggest that the presence or absence of an anti-feedant protein may not be a critical factor for the development of aphid nymphs if the anti-feedant protein is expressed in a high quality leaf substrate. However, it is also possible that there is an underlying concentration effect. Since low volumes of high quality phloem would also mean that the ingested dose of

GNA would be reduced and therefore possibly below a threshold of activity as seen in the potato leaf hopper, *Empoasca fabae* (Harris) (Habiba *et al.*, 1993).

In support of the above speculation, detoxification of GNA is unlikely for *A. solani* because aphids, unlike the brown plant hopper or green leafhopper, are not equipped with the appropriate gut enzymes (Powell *et al.*, 1993). For example, GNA has been detected in *M. persicae* honeydew indicating that digestion does not occur (Shi *et al.*, 1994). The absence of a toxic or anti-feedant effect on the first generation presented in this chapter is therefore related to an additional factor like high phloem quality that compensates for the presence of anti-feedant proteins (GNA and the truncated GNA protein). A similar relationship between the toxicity of proteinase inhibitors, protein quality and insect growth was observed by Broadway and Duffy, (1988), who noted that diets containing highly nutritious protein reduced the toxicity of soybean trypsin inhibitor to *Spodoptera exigua* larvae (Noctuidae). However, further research would be necessary to confirm the underlying mechanism of this effect.

The mean weights of the A1 adult aphids reared on leaves containing GNA increased from 1.32 mg ±0.077 at day 7 (bioassays 1 and 2) to 1.70 mg ±0.171 at day 12 (bioassay 3). As the weight data from day 7 and day 12 were recorded from different bioassays, it is difficult to ascertain the reasons why this occurred. Improved performance related to a change in phloem quality at the onset of senescence is probably not responsible for the weight increase as this response would not have been isolated to the TRV-GNA treatment. However, the apparent differences could be related to the growth stimulating properties of GNA when present in the diet at low concentrations (Sauvion *et al.*, 1995). The blots in Figure 6.2, and the time course experiments carried out in Chapter 4 confirm that the level of virally expressed GNA detected in leaf extracts decrease over the bioassay period and may show the transition of GNA from toxic molecule to stimulant. Future studies might address this mechanism by measuring honeydew production over the bioassay experiment (Powell *et al.*, 1995).

6.4.3 Nymph performance

The results from the study of the second nymph generation indicated that GNA delivered by the virus vector had anti-feedant properties for these nymphs despite having little apparent effect on the adult female's short-term fecundity. Statistical analysis revealed that both the virally expressed GNA and truncated GNA protein ($p < .01$ and $p < .001$ respectively) caused nymph antibiosis (a negative effect on larval development). The results from this part of the study are consistent with the significant inhibition of growth previously detected using image analysis of *A. solani* nymphs when reared on an artificial diet containing GNA (Down *et al.*, 1996), and in weight measurements for *M. persicae* fed on a similar diet (Sauvion *et al.*, 1996). It should also be noted that this part of the bioassay was performed on mature leaves, which supports the previous speculation that the stage of plant development may be an important factor in the data recorded from *in planta* bioassays.

The conflicting observations recorded for the first- and second-generation nymphs may be partially explained through maternal influence. In this case, adult female aphids feeding on phloem that contain lectins, because of parthenogenesis and the telescoping of generations, also expose pre-natal nymphs to this substance. Consequently, this could affect nymph performance and potentially affect the long-term fitness of the aphid colonists (reported in Kindleman and Dixon 1994). In the present study, all first generation nymphs were deposited by A0 aphids, which were reared on virus-free *N. benthamiana* plants. The maternal influence at this stage of the experiment would therefore be equivalent for all treatments. In contrast, adult A1 aphids were pre-conditioned on phloem that contained virus, GNA or the truncated GNA protein. It is therefore possible to speculate that the ingestion of GNA or truncated lectin by a female aphid could have a contributing detrimental effect on the fitness of pre-natal nymphs. Down *et al.*, (1996) also concluded that crops engineered to express GNA would not have an affect on *A. solani* until the second generation. However, a study by Hilder *et al.*, (1995) rearing *M. persicae* on tobacco expressing GNA over 6 generations found that GNA-conditioned aphids remained susceptible. Therefore, the virus-expression system could be adapted for future bioassays to study long-term effects, since there is evidence to suggest that the

pesticidal effect of proteins such as GNA may be obscured unless the studies are continued over several generations of pest organism.

6.4.4 Nymph antibiosis is related to high levels of GNA

For plants inoculated by TRV-GNA the quantity of GNA (ng) accumulated in the leaf tissue was quantified using densitometric analysis (Figure 6.3) and compared to nymph weight after 7 days. A trend of nymph antibiosis was detected over all 32 samples where performance was reduced as levels of GNA increased. However, nymph mortality, in this *A. solani* population was observed for leaf samples containing both high and low levels of accumulated GNA. This observation may suggest that variation in sensitivity to GNA does exist in clonal populations of *A. solani,* which highlights the importance of following the effects of GNA over consecutive generations and the potential for this aphid species to develop resistance towards GNA. However, this present data does show that virally expressed GNA accumulates at sufficiently high level in the phloem to act as an anti-feedant, although these levels may not reflect the amount of GNA ingested by the nymph. For example, larger nymphs may be related to the growth stimulatory effect of a sub-lethal dose of GNA rather than the ingestion of greater amounts of phloem. Similarly, nymphal antibiosis associated with higher levels of GNA may be related to an anti-feedant or antixenosis (negative feeding) effect or a combination of both effects. Antixenotic effects have previously been observed for *N. lugens* (Powell *et al.,* 1995a, 1998) and *M. persicae* (Gatehouse *et al.,* 1996). Future studies that would include measurements of the rate of honeydew production may clarify which factors are accountable for the observed antibiosis response.

It should be noted that while densitometric analysis was not as accurate a method of GNA quantification as an ELISA due to the imprecision of comparing autorads with different exposures and to the limited protein standards for comparison. The means of analysis is an indicator to how future results could be analyzed.

6.4.5 Truncated GNA is more toxic to *A. solani* than GNA

The toxic effect of the truncated GNA protein (expressed by TRV-fsGNA) was an unexpected observation as it was assumed that this protein, if expressed, would be

ineffective due to its monomeric form. The finding that the putative, truncated GNA protein had significantly greater anti-feedant properties than GNA toward the N2 nymphs was unexpected, particularly as similar negative effects were not observed in the nematode bioassays described in Chapter 5. Furthermore, this data suggests that the full-length GNA and the truncated GNA proteins have distinct biological effects that are distinguishable from each other using the virus expression system, after ingestion by *A. solani*. The fact that the truncated GNA protein's significant toxicity to the second generation of nymphs was also not evident for the first aphid generation during leaf development, may also support the hypothesis that high phloem quality may alleviate, or mask, anti-feedant properties to *A. solani*.

6.5 Conclusion

The results from this present series of insect bioassays demonstrate that the virus expression system is an undeveloped experimental tool that could be used to rapidly examine the affects of potential, bio-active proteins on *A. solani* colonization of plants.

1) Effects of virally expressed anti-feedants (GNA and truncated GNA) were detectable as differences in aphid responses at 7 and 12 days.

2) Differences in susceptibility between consecutive generations may be dependent on several contributing factors particularly phloem quantity and the multimechanistic effects of GNA (including maternal influences and negative feeding behaviour). The design of the virus bioassay system could be adapted to accommodate *in planta* behavioural studies or long-term physiological studies.

3) The truncated GNA protein has anti-feedant properties to *A. solani* suggesting that this molecule is a stronger resistance factor than GNA.

In conclusion, the results of the four aphid bioassays described in this study demonstrate that GNA, when derived from the TRV virus expression vector, produces results that are broadly similar to those from assays based on GNA-transgenic plants. Thus, the virus-expression bioassay could be developed to provide a more rapid, bioassay method for the investigation of pesticidal proteins.

Chapter 7

General Discussion

General Discussion

The first aim of this study was to characterize the dynamics of GFP expression by the TRV virus vector (Chapter 3). The second aim was to insert the snowdrop lectin (LECGNA2) into two virus-expression vectors based on TRV and PepRSV in place of the GFP gene, followed by the production of two mutants (a frame-shift and virus-only mutant) which would provide additional treatments against which the effects of GNA could be compared (see Chapter 2). These aims were successfully achieved. Data from time course experiments and tissue prints demonstrated that virally expressed proteins (GFP and GNA) accumulated rapidly in the leaves and roots (Chapter 4) which enabled the initiation of aphid and nematode bioassay experiments shortly after inoculation by the virus expression vector. This fulfilled the main objective of this project which was to develop a transient virus-based expression system that could provide a rational basis in which to rapidly screen primary gene products for anti-pathogenic properties. Data from nematode and aphid bioassays support the application of the virus expression system as a 'rapid' method of detecting anti-feedant properties since useful data was produced from short-term assays. For example, the aphid responses were recorded over a 12 day period, but affects were apparent after 7 days of exposure, which is a relatively short period of time compared to that of previous studies (Down *et al.,* 1996). The nematode responses were analyzed after 10 DAI (17 days total not including statistical analysis). The objectives of this research were successfully achieved despite fundamental differences between the test animals.

The third aim of this study was to perform nematode bioassays for the root knot nematode (*Meloidogyne* spp.). It was found that all treatments developed root galls indicating that no adverse effects were associated with the presence of GNA or truncated GNA for the nematode populations used in this assay. Consequently, no evidence was found to indicate that systemically accumulated GNA would equip plants with passive or active resistance and therefore does not have nematicidal properties. However, bioassays

did demonstrate that the presence of GNA was consistently associated with a decrease in galling (by 22%) and while the effects of truncated GNA were not significant, by process of elimination, the results did suggest that galling by the non-virulent *M. incognita* (present in the mixed nematode population) was inhibited. This was considered as an important finding as firstly, the experiments suggested that differences between virally expressed proteins, even when derived from the same sequence, could be detected efficiently in this system and secondly that variance within the nematode population could be identified.

Although the main objective of the virus expression system was to develop a rapid screening method, with hindsight the measurement of one parameter for the nematode bioassays was a limitation in this bioassay particularly as lectins are multifunctional molecules with potentially multiple functions or roles (Peumans *et al.*, 1995a,b). Ideally several parameters should have been measured as recommended by Adams and van Emden (1972). Presumably, future projects based on this system would consider the availability of resources (like labour), the level of known background information and time constraints. Thus the complexity of the design for planned nematode bioassays would reflect the short- or long-term research aim. For example, the measurement of galling might be acceptable for a rapid screening programme but anti-feedant effects might be missed if, as in the case of *A. solani*, they were only apparent in subsequent generations. In terms of information, it would have been more productive if the experiment had been extended to allow counts of galls with attached egg masses and the collection of egg masses for subsequent infections, which may have identified affects on development, fecundity and potential ovicidal properties.

In the long-term, these observations suggests that plants expressing nematicidal proteins may operate selectively in their effects on nematodes, or other animals in the rhizosphere, and consequently reduce variability in the soil community by removing susceptible individuals. However, the results also reflect the damage potential of this pest and the viability of the virus expression system for use in identification of other anti-nematicidal proteins.

The fourth aim to perform aphid bioassays for the Glasshouse potato aphid (*A. solani*) was successfully completed. Analysis of the aphid data indicated that the anti-feedant properties of GNA and the truncated protein were only detected during the development of the second nymph generation despite feeding from the same leaf. It was speculated that the differences in susceptibility between consecutive generations may be dependent on several contributing factors particularly phloem quantity and the multi-mechanistic effects of GNA including maternal influences and negative feeding behaviour. Although, future studies would be required to discriminate between the effects of lectin, plant growth stage (developing, mature or senescing leaves), antixenosis (negative feeding behaviour) and any maternal influence on the growth and development of subsequent generations of this aphid (for example, effect on symbiotic bacteria).

The observation that foreign protein degradation coincided with leaf maturation was initially recorded during the GFP and GNA time course experiments. This suggested that GNA proteolysis occurred during leaf ageing indicating that foreign proteins were susceptible to the increase in RNase and proteolytic enzymes that are associated with the processes of senescence (Huffaker, 1990; Noodén *et al.*,1997; Crafts-Brandner *et al.*, 1998). The significance of these observations was not realized until they were associated with the aphid bioassay data. For example, an important consideration for crop management strategies using transgenic plants would therefore be the initiation of additional pest control measures at the onset of leaf maturity when the resistance status of the plant, was in decline due to GNA proteolysis. This observation may have implications for the use of transgenic plants in the future. Since, although plants expressing anti-feedant protein would require additional protection against aphids at the later stages of plant development, the fact that the anti-feedant protein is being naturally degraded by the plant prior to harvesting may be acceptable to the public. This feature of trangenic crops would also support their utility in IMP strategies.

Recognition of the frameshift GNA protein by both nematodes and aphids formed the basis for the proposal that the frameshift GNA sequence coded for a stable truncated GNA protein. Although it was an unexpected observation that the truncated GNA protein provided a stronger resistance factor to the host plant compared to its parent protein, which suggests that truncated lectins may be a more obvious choice for future insecticidal proteins. Further studies would be required to determine whether the increased toxicity of the protein derived from frame-shifted GNA sequence, relative to the native GNA protein, was isolated to this species of aphid or extends to other organisms. Presumably the truncated protein had an increased specificity to an aphid receptor due to a higher affinity or specificity to an unknown receptor molecule induced due to changes in size and surface architecture. There is therefore scope for further research into evaluating the anti-feedant effects of fragments of proteins or lectins. However, the observation that these proteins may be species specific emphases the difficulties associated with engineering resistance to pests and the potential for selecting resistant organisms.

In conclusion, the results obtained from the current aphid bioassays confirm the view that the relationship between the aphid and its environment is complex.

Future research

There are several potential applications for the virus expression system;

1) Future experiments would be required to determine whether the virus system could be adapted to test the effect of pyramid gene expression in plant (Gatehouse *et al.*, 1993). For example, the infection of existing transgenic cultivars

2) Large numbers of insects exposed to anti-feedant proteins could be produced for extensive physiological, tri-trophic or long-term studies.

3) The virus expression system complies with the suggestion made by Mueller and Noling (1996) that more detailed reports are required for the implementation of management strategies, since this system is both rapid and cost effective in terms of time and finances. Presumably, it would also be possible to carry out nematode bioassays on collected soil samples enabling an advanced evaluation of nematode populations prior to the planting of transgenic lines and therefore enabling optimization of pest management strategies. It may also be possible to extend the bioassay system and combine the virus expression system with other control agents or soil amendments and therefore explore alternative control strategies.

The virus expression system is therefore potentially a powerful tool that could be used to explore areas of both insect and nematode management with relatively little investment in terms of effort and cost.

References

Adams, J.B. and van Emden, H.F. (1972) The biological properties of aphids and their host plant relationships In: Aphid Technology (Eds) H.F. van Emden Academic Press London and New York pp 48 -97

Alberts , B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989) Macromolecules: Structure, Shape, and Information In: Molecular Biology of the Cell. Second Edition. Garland Publishing, Inc. New York pp 87 - 132

Allsopp, P.G. and McGhie, T.K. (1996) Snowdrop and wheatgerm lectins and avidin as antimetabolites for the control of sugar whitegrubs. *Entomologia Experimentalis et Applicata,* 80, 409-414

An, G. (1985) High efficiency transformation of cultured tobacco cells. *Plant Physiology* 79, 568-570

Angenent, G.C. Posthumus, E. Brederode, F.T. and Bol, J.F. (1989a) Genome structure of *Tobacco Rattle Virus* strain PLB: further evidence on the occurrence of RNA recombination among tobraviruses. *Virology* 171 (1), 271-4

Angenent, G.C. Posthumus, E. and Bol, J.F. (1989b) Biological activity of transcripts synthesized *in vitro* from full length and mutated DNA copies of *Tobacco Rattle Virus* RNA 2. *Virology* 173, 68-76

Atkinson, H.J., Urwin, P.E., Lilley, C.J. and McPherson, M.J. (?) Genetically engineered resistance to nematodes
Genetical engineered resistance to nematodes ICPP Conference.

Atkinson, H.J., Unwin, P.E., Hansen, E. and McPherson, M.J. (1995) Designs for engineered resistance to root-parasitic nematodes. *Trends in Biotechnology* 13, 369-374

Auclair, J.L. (1963) Aphid feeding and nutrition. *Annual Reveiw of Entomology* 8, 439-90

Barker, K.R. (1985) Chapter 2 Nematode extraction and bioassays. In: An Advanced Treatise on *Meloidogyne* Volume II: Methodology. (Eds) K.R. Barker, C.C. Carter, and Sasser, J.N. A Cooperative Publication of the Department of Plant Pathology and the United States Agency for International Development. North Carolina pp19-35

Barker, K.R., Schmitt, D.P. and Imbriani, J.L. (1985) Chapter 10 Nematode population dynamics with emphasis on determining damage potential to crops. In An Advanced Treatise on

Meloidogyne Volume II: Methodology. (Eds) K.R. Barker, C.C. Carter, and Sasser, J.N. A Cooperative Publication of the Department of Plant Pathology and the United States Agency for International Development. North Carolina pp 135-148

Barre, A., van Damme, E.J.M., Peumans, W.J. and Rouge, P. (1996) Structure-function relationship of monocot mannose-binding lectins. *Plant Physiology* 112:1531-1540

Baulcombe, D.C., Chapman, S. and Santa Cruz, S. (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *The Plant Journal* 7(6),1045-1053

Bethke, J.A., Redak, R.A. and Schuch, U.K (1998) Melon aphid performance on chrysanthemum as mediated by cultivar, and differential levels of fertilization and irrigation. *Entomologia Experimentalis et Applicata* 88 (1), 41-47

Bird, AF. (1960) The effect of some single element deficiencies on the growth of *Meloidogyne javanica. Nematologica.* 5, 78-85

Bird, A.F. (1988) Cuticle printing of nematodes. *International Journal of Parasitology* 18 (6), 869-871

Bird, A.F., Bonig, I. and Bacic, A. (1989) Factors affecting the adhesion of microorganisms to the surfaces of plant-parasitic nematodes. *Parasitology,* 98(1): 155-164

Bocarra, M., Hamilton, W. Baulcombe, D. (1986) Gene organization at the end of *Tobacco rattle Virus. Journal of Cell Biochemistry* 38-38 Suppl. 10C

Bohlool, B. B. and Schmidt, E. L. (1974) Lectins: a possible basis for the specificity in the Rhizobium-legume root nodule symbiosis. *Science,* 185-269-71

Bouton, J. H., Hussey, R.S. and Smith, S. R. (1989) Greenhouse screening of alfalfa cultivars for resistance to southern root-knot nematode. *Crop Science,* 29(3): 823-825

Brandley, B.K and Schnaar R.L J. (1986) Cell-surface carbohydrates in cell recognition and response. *Journal of Leukocyte Biology* 40 (1), 97-111

Brierley. K. M., Goodman, B.A. and Mayo, M.A. (1993) A mobile element on a virus particle surface identified by nuclear magnetic resonance spectroscopy. *Biochemical Journal,* 293(3): 657-659

Broadway, R.M. and Duffy, S.S. (1988) The effect of plant protein quality on insect digestive physiology and the toxicity of plant proteinase inhibitors. *Journal of Insect Physiology Vol.* 34, No 12, 1111-1117

Broekaert, W. F., VanParijs, J., Leyns, F., Joos, H. and Peumans, W.J. (1989) A chitin binding lectin from stinging nettle rhizomes with anti-fungal properties. *Science* 245 (4922): 1100-1102

Brown, D.J.F., Ploeg, A.T. and Robinson, D.J. (1989) A review of reported associateions between *Trichodorus and Paratrichodorus* species (Nematoda: Trichodoridae) and tobraviruses with a description of a laboratory methods for examining virus transmission by trichodorids. *Revue Nematologie* 12, 235-241

Brown, D.J.F., Robertson, W.M., Neilson, R., Bem, F. and Robinson, D.J. (1996) Characterization and vector relation of a serologically distinct isolate of *Tobacco Rattle Virus* (TRV) transmitted by *Trichodorus similis* in Northern Greece. *European Journal of Plant Pathology* 102, 61-68

Brown, C.R., Mojtahedi, H., Santo, G.S. and Williamson, V.M. (1997) Effect of the *Mi* gene in tomato on reproductive factors of *Meloidogyne chitwoodi* and *M. hapla. Journal of Nematology* 29 (3), 416-419

Burrows, P.R. (1996) Modifying resistance to plant-parasitic nematodes. In: Genetic Engineering of Crop Plants for Resistance to Pests and Diseases. (Eds.) W.S. Pierpoint and P.R. Shewry. The British Crop Protection Council. Farnham. Surrey pp 38-43

Bustamanete, P.I. and Hull, R. (1998) Plant virus expression strategies. *Electronic Journal of Biotechnology* 0717-3458

Carpita, N.C. and Gibeaut, D.M. (1993) Structural models of primary-cell walls in flowering plants – consistency of molecular- structure with the physical-properties of the walls during growth. *Plant Journal* 3(1), 1-30

Casper, S.J. and Holt, C.A. (1996) Expression of the green fluorescent protein-encoding gene from a tobacco mosaic virus-based vector. *Gene* 173 (1 Spec No), 69-73

Chalfie, M., Tu. Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. (1994) *Science* 263, 802-5

Chen, C.-C., Chen, L.-L., Kuan, C,-C., Hou, R.F. (1981) Studies on the intracellular yeast-like symbiote in the brown plant hopper, *Nilparvata lugens* Stal.I. Histological observations and population changes of the symbiotes. *Z. ang. Ent.* 91: 321-327

Chiang, A. S., Yen, D. F. and Peng, W. K. (1986) Defence reaction of midgut epithelial cells in the rice moth larvae (*Corcyra cephalonica*) infected with *Bacillus thuringiensis. Ournal of Invertebrate Pathology*

Chapman, R.F. (1978) Feeding In: The Insects – Structure and Function. Hodder and Stouton. London pp 21-39

Clark, C. A. and Moyer, J.W. (1988) Compendium of sweet potato diseases. The American Phytopathological Society Press, St. Paul Minnesota. P 75

Cody, C.W., Prasher, D.C., Westler, W.M., Pendergast, F.G. and Ward, W.W. (1993) Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. *Biochemistry* 32, 1212-1218

Cook, G.M.W. (1986) Cell-surface carbohydrates – molecules in search of a function. *Journal of Cell Science* 45-70 Suppl. 4

Crafts-Brandner, S.J., Holzer, R. and Feller, U. (1998) Influence of nitrogen deficiency on senscence and the amounts of RNA and protein in wheat leaves. *Physiologia Plantarum* 102, 192-200

Czapla, T.H. and Lang, B.A. (1990) Effect of plant lectins on the larval development of European corn borer (Lepidoptera: Pyralidae) and Southern corn rootworm (Coleopetera: Chrysomelidae), *Journal of Economic Entomology* 83, 280-285

Davide, R.G. and Triantaphyllou, AC (1967) Influence of the environment on development and sex differentiation of the root-knot nematodes. *Nematologica*, 13, 102-110

Davis, L., Kaplan, D.T., Dickson, D.W., Mitchell, D.J. and Permar, T.A. (1988) Characterization of carbohydrates on the surface of second stage juveniles of *Meloidogyne. Journal of Nematology* 20 (4), 609-619

Davis, L., Kaplan, D.T., Dickson, D.W. and Mitchell, D.J. (1989) Root tissue response of two related soybean cultivars to infection by lectin-treated *Meloidogyne* spp. *Journal of Nematology* 21 (2), 219-228

Davis, L., Kaplan, D.T. (1992) Lectin binding to aqueaous-soluble and body wall proteins from infective juveniles of *Meloidogyne* species. *Fundamental and Applied Nematology* 15 (3), 243-250

Davis, L. and Danks, C. (1993) Carbohydrate/proteins interactions between the cuticle of infective juveniles of *Meloidogyne incognita* and spores of the obligate hyperparasite *Pasteuria pentrans. Nematologica* 39 (1), 53-64

de Ponti, O.M.B. and Mollema, C. (1992) Emerging breeding strategies for insect resistance. In: Plant Breeding in the 1990s. (Eds.) HT. Stalker and J.P. Murphy. CAB International UK pp 323-346

Devonshire, A.L. (1989) Insecticide resistance in *Myzus persicae* from field to gene and back again. *Pestic Biochem Phys* 34 (2), 174-178

Devonshire, A.L. and Rice, A.D. (1988) Aphid Bioassay Techniques. In Aphids, their biology, natural enemies and control. Vol B Eds. A. K. Minks and P Harrewijn. Amsterdam: Elsevier pp 119-128

Dixon, A.F.G. (1998) Chapter 3 Host Specificity and speciation In Aphid Ecology - An optimization approach. 2nd Edition. Chapman and Hall London pp 27 -58

Dixon, A.F.G. (1985) Structure of aphid populations *Annual Review of Entomology* 30, 155-174

Dixon, R.A. (1999) Plant natural products: the basis of biosynthetic diversity. *Current Opinion in Biotechnology* 10, 192-197

Dixon, R.A. and Lamb, C.J. (1990) Molecular communication in interactions between plants and microbial pathogens. *Annual Revue of Plant Physiology,* 41, 339-367

Dong, K., Barker, K.R. and Opperman, C.H. (1997) Genetics of soybean -*Heterodera glycines* interactions. *Journal of Nematology* 29 (4), 509-522

Douglas, A.E. and Prosser, W.A. (1992) Synthesis of the essential amino acid tryptophan in the pea pahid (*Acyrthosiphon pisum*) symbiosis. *Journal of Insect Physiology* 38(8), 565-568

Douglas, A.E. (1990) Nutritional interaction between *Myzus persiace* and its symbionts. In Aphid-Plant Genotype Interactions. Eds Campbell, R. K. and Eikenbary, R. D.) Elsevier Amserdam pp 319-328

Down, R.E., Ford, L., Woodhouse, S.D., Raemaekers, R.J.M., Leitch, B., Gatehouse, J.A. and Gatehouse, A.M.R. (2000) Snowdrop lectin (GNA) has no acute toxic

effects on a beneficial insect predator, the 2-spot ladybird (*Adalia bipunctata* L.) *Journal of Insect Physiology,* 46 (4), 397-391

Down, R.E., Gatehouse, A.M.R., Hamilton, W.D.O. and Gatehouse, J.A. (1996) Snowdrop lectin inhibits development and decreases fecundity of the glasshouse potato aphid (*Aulacorthum solani*) when administered in vitro and via transgenic plants both in laboratory and glasshouse trials. *Journal of Insect Physiology,* 42 (11-12), 1035-1045

Dozolme, P, Martymazars, D., Clemencet, M.C. and Marty, F. (1995) Monoclonal antibody TEM-106 reacts with a tonoplast intrinsic protein of 106 kDa from *Brassica oleraceae. Journal of Cell Science,* 108, 1509-1517, part 4

Du J, Foissac, X., Carss, A., Gatehouse, AMG and Gatehouse, JA (2000) Ferritin acts as the most abundant biunding protein for snowdrop lectin in the midgut of rice brown plant hopper (*Nilaparvata lugens*) *Insect Biochemistry and Molecular Biology*, Apr, 30: 4, 297-305

Edwards, P. J. (1989) Insect herbivory and plant defence theory. In: Toward a more exact ecology (eds P.J. Grubb and J.B. Whittaker) Blackwell Scientific Publications, Oxford, pp 275-297

Eisemann, C.C., Donaldson, R.A., Pearson, RD., Cadagon, L.C., Vuocolo, T. and Tellam, R.L. (1994) Larvividal activity of lectins on *Lucila cuprina*: Mechanism of action. *Entomologia Experimentalis et Applicata,* 72, 1-11

Engelhard, E.K. and Volkman, L.E. (1995) Developmental resistance in 4th-instar *Trichoplusia ni* orally inoculated with *Autographa californica* M nuclear polyhedrosis virus. *Virology,* 209 (2), 384-389

Etzler, M.E. (1985) Plant lectins: Molecular and biological aspects. *Annual Review of Plant Physiology* 36, 209-34

Evert, R.F., Eschrich. W,. Medlar, J.T. and Alfieri, F.J. (1968) Observations on penetration of linden branches by stylets of the aphid *Longistigma caryae. American Journal of Botany,* 55, 860-74

Fargette, M., Davies, K.G., Robinson, M.P. and Trudgill, D.L. (1994) Characterization os resistance breaking *Meloidogyne incognita*: Like populations using lectins, monoclonal antibodies and spores of *Pasteuria penetrans. Fundamental and Applied Nematology* 17(6), 537-542

Feeny, P. P. (1976) Plant apparency and chemical defences. *Recent Advances in Phytochemistry*, 10, 1-41

Federici, B. A. (1993) Insecticidal bacterial proteins identify the midgut epithelium as a source of novel target sites for insect control. *Archives of Insect Biochemistry and Physiology,* 22, 367-371

Fenton B, Stanley K., Fenton S, and Bolton-Smith C (1999) Differential binding of the insecticidal lectin GNA to human blood cells. *Lancet,* 354 (9187), 1354-1355 Oct 16

Figlerowicz, M. (2000) Role of RNA structure in non-homologous recombination between genomic molecules of Brome Mosaic Virus. *Nucleic Acids Research,* 28 (8), 1714-1723

Foissac, X., Loc, N. T., Christou, P., Gatehouse, A.M.R. and Gatehouse, J.A. (2000) Resistance to green leafhopper (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*) in transgenic rice expressing snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) *Journal of Insect Physiology,* 46, 573-583

Fulton, R. W. (1986) Practices and precautions in the use of cross protection for plant-virus disease control. *Annual Review of Phytopathology,* 24, 67-81

Gabius, H.J. (2000) Biological information transfer beyond the genetic code: The sugar code. *Naturwissenschaften,* 87 (3), 108-121

Gabius, H.J. (2001) Glycohistochemistry: The why and how of detection and localization of endogenous lectins. *Anatomia Histologia Embryologia- Journal of Veterinary Medicine Series C,* 30 (1), 3-31

Gatehouse, A.M.R., Dewey, F.M., Dove, J., Fenton, K.A. and Pusztai, A.J. (1984) Effect of seed lecctins from *Phaseolus vulgaris* on the development of larvae of *Callosobruchus maculatus*; mechanism of toxicity. *Journal of Science Food and Agriculture.* 35, 373-380

Gatehouse, A.M.R., Shackley, S.J., Fenton, K., Brydon, J. and Pusztai, A. (1989) Mechanism of seed tolereance by major insect storage pest of *Phaseolus vulgaris, Ascanthoscelides obtectus. Journal of Science Food and Agriculture,* 47, 269-280

Gatehouse, A.M.R., Barbieri, L., Stirpe, F. and Croy, R.R.D. (1990) Effects of ribosome inactivating proteins on insect development- differences between Lepidoptera and Coleoptera. *Entomologia Experimentalis et Applicata,* 54, 43-51

Gatehouse, A.M.R., Boulter D. and Hilder V.A. (1992) Potential of plant derived genes in the genetic manipulation of of crops for insect resistance. In: Plant Genetic Manipulation for Crop Protection (Eds Gatehouse AMR., Hilder VA and Boulter D) pp 135-153

Gatehouse, A.M.R., Shi, Y., Powell K., Brough C., Hilder VA., Hamilton WDO., Newell CA.,
Merryweather A., Boulter D and Gatehouse, J.A. (1993) Approaches to insect resistance using transgenic plants. . *Philosophical Transactions of the Royal Society of London Series* B, 342, 279-286

Gatehouse, A.M.R. Hilder, V.A. and Powell, K.S. (1994) Insect-resistant transgenic plants – choosing the gene to do the job. *Biochemistry Society T,* 22 (4), 944-949

Gatehouse, J.A., Gatehouse, A.M.R. and Powell, K.S. (1994) Novel genes for the production of insect-resistant transgenic plants. *Journal of Cell Biochemistry,* 75-75 Suppl. 18A Jan 4

Gatehouse, A.M.R., Down, R.E., Powell, K.S., Sauvion, N., Rahbé, Y., Newell, C., Merryweather, Hamilton, W.D.O., and Gatehouse, J.A. (1996) Transgenic potato plants with enhanced resistance to the peach-potato aphid *Myzus persicae Entomologia Experimentalis et Applicata,* 79, 295-307

Geibel, J. (1982) Mechanisms of resistance of plant nematodes. *Annual Review of Phytopathology,* 20, 257-79

Goddijn, O.J.M., Lindsey, K., van der Leer, F.M., Klap, J.C. and Sijmons, P.C. (1993) Differential gene expression in neamtode-induced feeding structures of transgenic plants harbouring promoter-*gus*A fusion constructs. *The Plant Journal,* 4 (5), 863-873

Goldstein, I. J. and Poretz, R.D. (1986) Isolation, physiochemical characterization and carbohydrate binding specificity of lectins, In: The Lectins: Properties, Functions and Applications in Biology and Medicine. (Eds)Liener, I.E., Sharon, N. and Goldstein, I.J. pp. 33-248, New York Academic Press.

Grimes, H.D. and Breidenbach, R.W. (1987) Plant plasma-membrane proteins – immunological characterization of a major 75 kilodalton protein group. *Plant Physiology,* 85 (4), 1048-1054

Guldemond, J.A., van den Brink, W.J. and den Belder, E. (1998) Methods of assessing population increase in aphids and the effect of growth stage on host plant population growth rates *Entomologia Experimentalis Applicata,* 86, 163-173

Gupta, R. and Sharma, N.K. (1991a) A study of the nematicidal activity of alicin-an active principle in garlic, *Allium sativaum* L., against root-knot nematode, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949. *International Journal of Pest Management,* 39 (3), 390-392

Gupta, R. and Sharma, N.K. (1991b) Nematicidal properites of garlic, *Allium sativum* L. *Indian Journal of Nematology* 21 (1), 14-18

Habibi, J., Backus, E. and Czapla, T. (1993) Plant lectins affect survival of the potato leafhopper (Homoptera: Cicadellidae) *Journal of Economic Entomology,* 86, 945-951

Halbrendt, J.M. (1996) Allelopathy in the management of plant-parasitic nematodes. *Journal of Nematology,* 28 (1), 8-14

Hamilton, W.D.O., Boccara, M., Robinson, D.J. and Baulcombe, D.C. (1987) The complete sequence of *Tobacco Rattle Virus* RNA 1. *Journal of General Virology,* 68, 2563-2575

Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science,* 286, 950-953

Harrewijn, P. (1990) Resistance mechanisms of plant genotypes to various aphid species. In: Aphid-Plant Genotypes Interactions (Eds) R K Campbell and ED Eikenbary. Elsevier Amsterdam: pp117-130

Harrison, B.D. (1958) Relationship between Beet Ringspot, Potato Bouquet and Tomato Black Ring viruses. *Journal of General Microbiology,* 18, 450-460

Harrison, B.D. and Woods (1966) Serotypes and particle dimensions of *Tobacco Rattle Viruses* from Europe and America. *Virology,* 28 610-620

Harrison, F.L. and Chesterton, C.J. (1980) Factors mediating cell-cell recognition and adhesion – galaptins, a recently discovered class of bridging molecules. *FEBS lett* 122 (2), 157-165

Harrison, B.D. and Robinson, D.J. (1982) Genomic reconstitution and nucleic acid hybridization as methods of identifying particle-deficient isolates of *Tobacco Rattle Virus* in potato plants with stem-mottle disease. *Journal of Virology Methods,* 5 (5-6), 255-65

Harrison, B.D. and Robinson, D.J. (1996) Tobacco rattle tobravirus, 1984. In Viruses of plants. Descriptions and lists from the VIDE database. Eds. A.A. Brunt,. K. Crabtree., M.J. Dallwitz., A.J. Gibbs, ans L. Watson. CAB International UK pp 1263 - 67

Harper, S.M., Crenshaw, R.W., Mullins M.A. and Privalle, L.S. (1995) Lectin binding to insect brush border membrane. *Journal of Economic Entomology,* 88(5), 1197-1202

Harper, B.K., Mabon, S.A., Leffel, S.M., Halifhill, M.D., Richards, H.A., Moyer, K.A. and Stewart Jr, C.N. (1999) Green fluorescent protein as a marker for expression of a second gene in transgenic plants. *Nature Biotechnology,* 17, 1125-1129

Hartman, K.M. and Sasser, J.N. (1985) Chapter 5 Identification of *Meloidogyne* sppecies on the basis of differential host test and perineal-pattern morphology. In An Advanced Treatise on *Meloidogyne* Volume II: Methodology. (Eds) K.R. Barker, C.C. Carter, and Sasser, J.N. A Cooperative Publication of the Department of Plant Pathology and the United States Agency for International Development. North Carolina pp 69 - 77

Hasselhoff, J., Siemering, K.R., Prasher, D.C. and Hodge, S. (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proceedings of the National Academy of Science USA,* 94, 2122-2127

Heim, R., Prasher, D.C. and Tsien, R.Y. (1994) Wavelength mutations and posttrannslational autoxiadation of green fluorescent protein. *Proceedings of the National Academy of Science USA* 91, 12501-12504

Herbes, K. and Sonnewald, U. (1999) Production of new/modified proteins in transgenic plants. *Current Opinions in Biotechnology* 10 (2), 163-8

Hernández, C., Mathis, A., Brown, D.J.F. and Bol, J.F. (1995) Sequence of RNA2 of a nematode-transmissible isolate of *Tobacco Rattle Virus. Journal of General Virology* 76, 2847-2851

Hernández, C., Carette, J.E., Brown, D.J.F. and Bol, J.F. (1996) Serial passage of *Tobacco Rattle Virus* under different selection conditions results in deletion of structural and nonstructural genes in RNA2. *Journal of Virology* 70 (8), 4933-4940.

Hernández, C.,Visser, P.B., Brown, D.J.F. and Bol, J.F. (1997) Transmission of *Tobacco Rattle Virus* isolate PpK20 by its nematode vector requires one of the two non-structural genes in the viral RNA 2. *Journal of General Virology* 78, 465-467

Hester, G., Kaku, H., Goldstein, I.J. and Schubert-Wright C. (1995) Structure of mannose-specific snowdrop (*Galanthus nivalis*) lectin is representative of a new plant lectin family. *Nature Structural Biology* Volume 2 (6),472-479

Hester, G. and Wright, C.S. (1996) The mannose specific bulb lectin from *Galanthus nivalis* (snowdrop) binds mono- and dimannosides at distinct sites. Structure analysis of refined complexes at 2.3 angstrom and 3.0 angstrom resolution. *Journal of Molecular Biology* 262(4), 516-531

Hilder, V.A., Powell, K.S., Gatehouse, A.M.R., Gatehouse, J.A., Gatehouse, L.N. Shi, Y., Hamilton, W.D.O., Merryweather, A., Newell, C., Timans, J.C., Peumans, W.J., van Damme, E.J.M. and Boulter, D. (1995) Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids. *Transgenic Research* 4, 18-25

Hill, D.S. (1987) Agricultural Insect pests of temperate regions and their control, pp 216. Cambridge University Press, Cambridge

Hester, G., Kaku, H., Goldstein, I.J. and Schubert Wright,C. (1995) Structure of mannose-specific snowdrop (*Galanthus nivalis*) lectin is representative of a new plant lectin family. *Nature Structural biology* 2(6),472-479

Hester, G. and Schubert Wright, S. (1996) The mannose-specific bulblectin from *Galanthus nivalis* (Snowdrop) binds mono- and dimannosides at distinct sites. Structure analysis of refined complexes at 2.3 A and 3 A resolution. *Journal of Molecular Biology* 262. 516-531

Heusing, J. E., Murdock, L. L. and Shade, R. E. (1991) Rice and stinging nettle lectins; insecticidal activity similar to wheat germ agglutinin. *Phytochemistry,* 30: 3565-3568

Hoover, K., Washburn, J.O. amd Volkman, L.E. (2000) Midgut-based resistance of *Heliothis virescens* to baculovirus infection mediated by phytochemicals in cotton. *Journal of Insect Physiology* 46, 999-1007

Horsch, R.B. (1993) Commercialization of genetically engineered crops. *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences* 342 (1301), 287-291

Huffaker, R.C. (1990) Proteolytic activity during senscence of plants. *New Phytologist Tansley Review No 25* 116, 199-231

Hussey, R. S. and Boer, H. R. (1981) A greenhouse screening procedure for root-knot nematode resistance in soybeans. *Crop Science,* 21(5): 794-796

Ibrahim, S.K. (1991) Distribution of carbohydrates on the cuticle surface of several development stages of *Meloidogyne javanica. Nematologica* 37 (3), 275-284

Ibrahim, I.K.A. and Rezk, M.A. (1986) The root-knot neamtodes – A major problem in crop production in Egypt. In: Advances in plant nematology Magbool, MA., Abdul Gaffar, Mogan Golden, A., Krusbeg, L.R. (Eds) "Proceedings of the US-Pakistan International Workshop on Plant Nematology" April 6-8, 1986, Karachi, Pakistan. Pp 81-98

Jatala, P. (1986) Biological control of plant-parasitic nematodes. *Annual Review of Phytopathology.* 24,453-89

Jepson SB (1987) Identification of root-knot nematodes (*Meloidogyne* species) CAB International, Wallingford, UK pp265

Jones, M. G. (1981) Host cell responses to endoparasitic nematode attack: structure and function of giant cells and syncytia. *Annals of Applied Biology* 97, 353-372

Jones, M. G. (1981b) The development and function of plant cells modified by endoparasitic nematodes. In: Plant Parasitic Nematodes. (Eds) B.M. Zuckerman and A.R. Rhode. Vol. 3, pp 255-279

Jones, M.G.K. and Northcote, D.H. (1972) Multinucleate transfer cells induced in Coleus roots by root-knot nematode, *Meloidogyne arenaria. Protoplasma* 75, 381-395

Jones, M.G.K. and Payne, H.L. (1978) Early stages of nematode-induced giant cell formation in roots of *Impatiens balsamina. Journal of Nematology* 10: 70-84

Kaku, H., van Damme, E.J.M., Peumans, W.J., Goldstein, I.J. (1990) Carbohydrate-binding specificity of the daffodil (*Narcissus pseudonarcissus*) and amaryllis (*Hippeastrum* hybr.) bulb lectins. Arch. Biochem. Biophys. 279. 298-304

Kaku, H., and Goldstein, I.J. (1992) Interaction of linear mannose oligosaccharides with three mannose-specific bulb lectins. Comparisons with mannose/glucose binding lectins. *Carbohydrate Research.* 229, 337-346

Karanastasi, E., Vassilakos, N., Roberts, I.M., MacFarlane, S.A. and Brown, D.J.F. (2000) Immunogold localization of *Tobacco Rattle Virus* particles within *Paratrichodorus anemones. Journal of Nematology* 32 (1), 5-12

Kindlemann, P. and Dixon A.F.G. (1994) Evolution of host range in aphids. *European Journal of Entomology,* 91, 91-96

Kindlemann, P. and Dixon A.F.G. (1989) Development constraints in the evolution of reproductive strategies: telescoping of generations in parthenogenetic aphids. *Functional Ecology*, 3, 31-537

Laemmli, U. K. (1970) Cleaveage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685

Lai, M.M.C. (1992) RNA recombination in animal and plant viruses. *Microbial Revues* 62-79

Laine, R.A. (1997) Information capacity of the carbohydrate code. *Pure and Applied Chemistry*, 69 (9), 1867-1873

Legorburu, M.J., Robinson, D.J., and Torrance, L. (1996) Features of the *Tobbaco Rattle Virus* particle that are antigenic and sensitive to proteolytic digestion. *Journal of General Virology* 77, 855-859

Lilley, C.J., Devlin, F., Urwin, P.E. and Atkinson, H.J. (1999) Parasitic nematodes, proteases and transgenic plants. *Parasitology Today* 15(10), 414-417

Lin, H-J. and McClure, M.A. (1996) Surface coat of *Meloidogyne incognita. Journal of Nematology* 28 (2), 216-224

Lindbo, J.A., Silva-Rosales, L., Proesbsting, W. and Dougherty, W.G. (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resitiance. *The Plant Cell* 5, 1749-1759

Loeb, M.J. and Haskim, R.S. (1996) Insect midgut epithelium *In vitro:* an insect stem cell system. *Journal of Insect Physiology.* 42 (11-12), 1103-111

Lucas, W. J. and Gilbertson, R. L. (1994) Plasmodesmata in relation to viral movement within leaf tissues. *Annual Review of Phytopathology,* 32, 387-411

MacCullock, L.A. (1990) "Chemotactic and electrotactic localisation of plant roots by parasitic nematodes." Ph.D. thesis, University of Aberdeen, Scotland

MacFarlane S.A., Mathis, A. and Bol, J.F. (1994) Heterologous encapsidation of recombinant *Pea Early Browning Virus. Journal of General Virology* 75, 1423-1429

MacFarlane S.A., Brown, D.J.F. and Bol J.F. (1995) The transmission by nematodes of tobraviruses is not determined exclusively by the virus coat protein. *European Journal of Plant Pathology* 101, 535-539

MacFarlane, S.A. and Brown, D.J.F. (1995) Sequence comparison of RNA2 of neamtode transmissible and nematode non-transmissible isolates of pea early-browning virus suggests that gene encoding the 29 kDa protein may be involved in nematode transmission. *Journal of General Virology,* 76, 1299-1304

MacFarlane, S.A., Wallis C.V., and Brown D.J.F (1996) Multiple virus genes involved in the nematode transmission of *Pea Early Browing Virus. Virology,* 219, 417-422

MacFarlane, S.A. (1996) Rapid cloning uncharacterized Tobacco Rattle Virus isolates using long template PCR. *Journal of Virology Methods*, 56(1): 91-98

MacFarlane, S.A. (1999) Molecular biology of the tobraviruses. *Journal of General Virology* 80: 2799-2807

MacFarlane, S.A., Vassilakos, N. and Brown, D.J.F. (1999) Similarities in the genome organization of *Tobacco Rattle Virus* and pea early-browning virus isolates that are transmitted by the same vector nematode. 80:273-276

MacFarlane, S.A., and Popovich, A.H. (2000) Efficient expression of foreign proteins in roots from Tobravirus vectors. *Virology* 267, 29-35

Mankau, R. (1980) Biocontrol: Fungi as nematode control agents. *Journal of Nematology* 12(4) 244-252

Mansky, L.M. Andrews, R.E., Durand Jr, D.P. and Hill, J.H. (1990) Plant virus location in leaf tissue by press blotting. *Plant Molecular Biology Report* 8, 13-17

Marban-Mendoza, N., Jeyaprakash, A., Jansson, H, -B., Damon Jr, R.A. and Zuckerman, B.M. (1987) Control of root-knot nematodes on tomato by lectins. *Journal of Nematology* 19 (3), 331-335

Marban-Mendoza, N., Dicklow, M.B. and Zuckerman, B.M. (1989) Evaluation of control of *Meloidogyne incognita* and *Nacobbus aberrans* on tomato by two leguminous plants. *Revue de Nematologie* 12 (3), 257-264

Marban-Mendoza, N., Dicklow, M.B. and Zuckerman, B.M. (1992) Control of *Meloidogyne incognita* on tomato by two leguminous plants. *Fundamental and Applied Nematology* 15 (2), 97-100

Mayo, M.A. Brierley. K.M. and Goodman, B.A. (1993) Developments in the understanding of the particle structure of tobraviruses. *Biochemie* 75:639-644

Millam, S. (1991) Introduction to DNA isolation and analysis. Brighton Crop Protection Conference mono. No. 48 Molecular Biology - Its practice and role in crop protection pp 3 - 21

McClure, M.A. and Viglierchio, (1966) The influence of host nutrition and intensity of infection on the sex ratio and development of *Meloidogyne incognita* in sterile agar cultures of excised cucumber roots. *Nematologica*, 12, 248-258

McClure, M. A. and Stynes, B.A. (1988) Lectin binding on the aphidial exudates of *Meloidogyne. Journal of Nematology* 20 (2), 321-326

Mojtahedi, H. and Santo, G.S. (1999) Ecology of Paratrichodorus allius and its relationship to the corky ring-spot disease of potato in the Pacific Northwest. *American Journal of Potato Research* 76 (5) Sept.-Oct., :273-280

Morin, J.G. and Hastings, J.W. (1971) *Journal of Cell Physiology* 77:313-18

Mueller, A.M., Mooney, A.L. and MacFarlane, S.A. (1997) Replication of *in vitro* tobravirus recombinants shows that the specificity of template recognition is determined by 5' non-coding but not 3' non-coding sequences. *Journal of General Virology* 78, 2085-2088

Meuller, J. D. and Noling, J. W. (1996) Standardization of reporting procedures for nematicide efficacy testing: A research and extension perspective. *Journal of Nematology*, 28(4S), 575-585

Murdoch, L.L. Huesing, J.E. Nielsen, S.S. Pratt, R.C. and Shade, R.E. (1990) Biological effects of plant lectins on the cowpea weevil. *Phytochemistry* 29, 85-89

Mugniery, D. (1977) Post embryonic development in relation to temperature of *Globodera rostochiensis* and *G. pallida* (Nematoda:Heteroderidae) *Revue de Nematologie* 1, 3-12

Mugniery, D. and Balandras, (1980a) Mise au point d'une test de resistance précoce des pommes de terre à Globodera sp. *Potato Research* 29:45-53

Mugniery, D. and Balandras, (1980b) Test de résistance de descendence de pommes de terre à *Globodera rostochiensis*. *Potato Research* 29:131-139

Mugniery, D. and Balandras, (1980c) Influence du type de test et de la population de *Globodera pallida* Stone sur l'évaluation de la résistance des variétés de pomme de terre en insciption. *Potato Research* 32:311-320

Murphy, F.A., Fauquet, C.M., Bishop DHL., Ghabrial SA Jarvis AW Martelli GP Mayo MA and Sum MD (Eds.) (1995) Virus taxonomy: Classification and Nomenclature of viruses. Springer-Verlag, Wien.

Napper, C.E., Dyson, M.H. and Taylor, M.E. (2001) An extended conformation of the macrophage mannose receptor. *The Journal of Biological Chemistry* 276 (18), 14759-14766

Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. and Kobayashi, H. (1999) Non-invasive quantitative detectin and applications of non-toxic S56T-type green fluorescent protein in living plants. *Plant Journal* 18(4), 455-463

Niebel, A., Gheyson, G. and van Montau, M. (1994) Plant-cyst nematode and plant-root-knot neamtode interactions. *Parasitology Today,* 10 (11), 424-430

Noodén, L.J., Guiamét, J.J. and John, I. (1997) Senescence mechanisms. *Physiologia Plantarum* 101, 746-753

Nsimba-Lubaki, M. and Peumans, W.J., (1986) Seasonal fluctuations of lectin in bark of elderberry (*Sambucus nigra*) and black locust (*Robinia pseudocacia*) *Plant Physiology*, 80:747-51

Oda, Y. and Miniami, K. (1986) Isolation and characterization of a lectin from tulip bulbs, *Tulipa gesneriana* bulbs. *European Journal of Biochemistry* 159: 239-245

Ogallo, J.L., Goodell, P.B., Eckert, J. and Roberts, P.A. (1997) Evaluation of NemX, a new cultivar of cotton with high resistance to *Meloidogyne incognita*. *Journal of Nematology* 29 (4), 531-537

Pang, S.Z., DeBoer, D.L., Wan, Y., Ye, G., Layton, J.G., Neher, M.K., Armstrong., C.L., Fry, J.E., Hinchee, M.A. and Fromm, M.E. (1996) An improved green fluorescent protein gene as a vital marker in plants. *Plant physiology* 112(3), 893-900

Peumans, W.J. and Stinissen, H. M. (1983) Gramineae lectins: occurrence, molecular biology and physiological functional. In Chemical Taxonomy, Molecular Biological and Functional of Plants Lectins, ed. I. J. Goldstein, M. E. Etzler, pp 99-116 New York; Liss

Peumans, W.L. and van Damme, E.J.M. (1995a) Lectins as plant defence proteins. *Plant Physiology* 109:347-352

Peumans W.L. and van Damme, E.J.M. (1995b) Lectins as plant defense proteins. In Lectins Biomedical Perspectives. (Eds.) Arpad Pusztai and Susan Bardocz Taylor and Francis London pp 1-22

Peumans, W.J., Nsimba-Lubaki, M., Peeters, B. and Broekaert, W.F. (1985) Isolation and partial characterization ofa lectin from Aegopodium podagraria rhizomes, *Planta*, 146:75-82

Phillips, M.S., Trudgill, D.L. and Evans, K (1988) The use of single, spaced potato plants to assess their tolerance of damage by potato cyst nematodes. *Potato Research* 31:469-475

Ploeg, A.T., Asjes, C.J. and Brown, D.J.F (1991) Tobacco rattle serotypes and associated nematode vector species of Trichodoridae in the bulb-growing areas in the Netherlands. *Netherlands Journal of Plant Pathology* 97:311-319

Ploeg, A.T., Brown, D.J.F. and Robinson, D.J. (1992) The association between species of *Trichodorus and Paratrichodorus* vector nematodes and serotypes of *Tobacco Rattle Virus*. *Annals of Applied Biology* 121, 619-630

Ploeg, A.T., Mathis, A., Bol, J.F., Brown, D.J.F. and Robinson, D.J. (1993) Susceptibility of transgenic tobacco plants expressing *Tobacco Rattle Virus* coat protein to nematode-transmitted and mechanically inoculated *Tobacco Rattle Virus*. *Journal of General Virology* 74, 2709-2715

Ploeg, A.T., Robinson D.J. and Brown, D.J. (1993) RNA-2 of *Tobacco Rattle Virus* encodes the determinants of transmissibility by trichodorid vector nematodes. *Journal of general virology* 74 (7), 1463-6

Ponti, J, J. D., Cavada, B.S. and Silveira-Filho, J. (1996) Test with lectin in the control of *Meloidogyne incognita* on tomato. *Fitopatologica Brasieira,* 21(4), 489-491

Powell KS., Gatehouse, A.M.R., Hilder, V.A., and Gatehouse, J.A. (1993) Anti-metabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important

rice pests, *Nilaparvata lugens and Nephotettix cinciteps. Entomologia experimentalis et Applicata 66, 119-126*

Powell, K.S., Gatehouse, A.M.R., Hilder, V.A., and Gatehouse, J.A. (1995a) Anti-feedant effects of plant lectins and an enzyme on the adult stage of the rice brown planthopper *Nilaparvata lugens. Entomologia experimentalis et Applicata* 75, 51-59

Powell, K.S., Gatehouse, A.M.R., Hilder, V.A., van Damme, E.J.M., Peumans, W.J., Boonjawat, J., Horsham, K. and Gatehouse, J.A. (1995b) Different anti-metabolic effects of related lectins towards nymphal stages of *Nilaparvata lugens. Entomologia Experimentalis et Applicata* 75, 61-64

Powell, K.S., Spence, J., Bharanthi, M., Gatehouse, J.A. and Gatehouse, A.M.R. (1998) Immunohistochemical and developmental studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown planthopper, *Nilaparvata lugens* (Stal) *Journal of Insect Physiology* 44, 529-539

Prasher, D.C. Eckenrode, V.K., Ward, W.W., Prendergast, F.G. and Cormier, M.J. (1992) Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene* 111(2), 229-233

Pusztai,A., Ewen, S.W.B. and Grant, G., Peumans, W.J., Van Damme, E.J.M., Rubio, L. and Bardocz, S. (1990) Relationship between survival and binding of plant lectins during small intestinal passage and their effectiveness as growth factors. *Digestion* 46, 308-316, Suppl 2

Rahbé, Y., Sauvion, N., Febvay, G., Peumans, W.J. and Gatehouse, A.M.R. (1995) Toxicity of lectins and processing of ingested proteins in the pea aphid *Acyrthosiphon pisum. Entomologia Experimentalis et Applicata,* 76(2), 143-155

Rahbé, Y.and Febvay, G. (1993) Protein toxicity to aphids – an *in vitro* test on *Acyrthosiphon pisum. Entomologia Experimentalis et Applicata* 67 (2), 149-160

Rebois, R.V., Madden, P.A. and Eldridge, B.J. (1975) Some ultra-structural changes induced in resistant and susceptible soybean roots following infection by *Rotylenchus reniformis. Journal of Nematology* 7, 122-139

Reichel, C. and Beachy, R.N. (1998) *Tobacco Mosaic Virus* infection induces severe morphological changes of the endoplasmic reticulu. *Proceedings of the National Academy of Science USA* 95(19), 11169-74

Rizzuto, R., Brini, M., DeGiorgi, F., Rossi, R., Heim, R., Tsien, R.Y. and Pozzan, T. (1996) Double labelling of subcellular structures with organelle-targeted GFP mutants *in vivo*. *Current Biology* 6 (2), 183-88

Roberts, P. A. (1995) Conceptual and practical aspects of variability in root-knot nematodes related to plant resistance. *Annual Review of Phytopathology* (1995), 199-221

Roberts, A.G. (1999) "The movement of *Potato Virus X* in *Nicotiana benthamiana*" Ph.D. thesis, University of Dundee, Scotland

Roberts, A.G., Santa Cruz, S., Roberts, I.M., Prior, D.A.M., Turgeon, R. and Oparka, K.J. (1997) Phloem unloading in sink leaves of *Nicotiana benthamiana*: Comparison of a fluorescent solute with a fluorescent virus. *Plant Cell* 9 (8),1381-1396

Robertson, W.M., Spiegel, Y., Jansson, H.B., Marban-Mendoza, N. and Zuckerman, B.M (1989) Surface carbohydrates of plant parasitic nematodes. *Nematologica* 35, (2), 180-186

Robinson, D.J (1983) RNA species of *Tobacco Rattle Virus* strains and their nucleotide sequence relationships. *Journal of general virology* 64 657-665

Robinson, D.J and Harrison, B.D (1985) Unequal variation in the two genome parts of tobraviruses and evidence for the existence of three separate viruses. *Journal of general virology* 66:171-176

Robinson, D.J., Hamilton, W.D.O., Harrison, B.D. and Baulcombe, D.C. (1987) Two anomalous tobravirus isolates: Evidence for RNA recombination in nature. *Journal of General Virology* 68, 2551-2561

Robinson, D.J (1996) *Pepper Ringspot Tobravirus,* 1987 In Viruses of plants. Descriptions and lists from the VIDE database. Eds. A.A. Brunt,. K. Crabtree., M.J. Dallwitz., A.J. Gibbs, and L. Watson. CAB International UK pp 955-57

Salisbury, F. B. and Ross, C. W. (1992) Chapter 1: Plant physiology and plant cells. In Plant Physiology. Fourth Edition. Eds. J. C. Carey. Wadsworth California

Sambrooke, J., Fritsch, E.F., and Maniatis, T.A. (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sasser, J.N., Eisenback, J.D. and Carter, C.C. (1983) The international *Meloidogyne* project – its goals and accomplishments. *Annual Review of Phytopathology* 21, 271-88

Sasser, J.N. and Freckman, D.W. (1987) A world perspective on nematology: the role of the society In Vistas on Nematology. Eds. Veeck, J.A., Dickson, D.W. and Hyattsville MD: Society of Nematologists pp7-14

Sauvion, N., Rahbé, Y., Peumans, W.J., van Damme, E.J.M., Gatehouse, A.M.R., and Gatehouse, J.A. (1996) Effects of GNA and other mannose binding lectins on development and fecundity of the peach-potato aphid *Myzus persicae. Entomologia Experimentalis et Applicata,* 79, 285-293

Savka, M.A., Ravillion, B., Noel, G.R. and Farrand, S.K. (1990) Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode. *Phytopathology* 80 (5), 503-508

Sayre, R. M. (1986) Pathogens for biological control of nematodes. *Crop Protection,* 5(4): 268-276

Schmitt, C., Mueller, A.-M,. Mooney, A., Brown, D.J.F., and Macfarlane, S.A. (1998) Immunological detection and mutational analysis of the RNA2-encoded nematode transmission proteins of *Pea Early Browning Virus. Journal of General Virology* 79, 1281-1288

Schmitt, D.P. and Shannon, G. (1992) Differentiating soybean responses to *Heterodera glycines* races. *Crop Science* 32, 275-277

Scholte, K. (1990) Causes of differences in growth pattern, yield and quality of potatoes (*Solanum tuberosum* L.) in short rotations on sandy soil as affected by crop rotation, cultivar and application of granular nematicides. *Potato research* 33, 181-190

Scholthof, H.B., Scholthof, K,B., and Jackson, A.O. (1996) Plant virus gene vectors for transient expression of foreign proteins in plants. *Annual Review of Phytopathology* 34, 299-323

Schmitt, D.P. and Shannon, G. (1992) Differentiating soybean responses to *Heterodera glycines* races. *Crop Science* 32(1), 275-277

Sharon, N. and Lis, H. (1989) Lectins as cell recognition molecules. *Science* 246:227-234

Shi, Y., Wang, M.B., Powel K,S., van Damme, E., Hilder, V.A., Gatehouse, A.M.R., Boulter, D. and Gatehouse, J.A. (1994) Use of the rice sucrose synthase-1 promoter to direct phloem-specific expression of β-glucuronidase and snowdrop lectin genes in transgenic tobacco plants. *Journal of Experimental Biology* 45(274), 623-631

Shi, Y., Wang M.B., Powell, K.S., van Damme, E., Hilder VA., Gatehouse, A.M.R., Boulter, D. and Gatehouse, J.A. (1993) Use of the rice sucrose synthase-1 promoter to direct phloem-specific expression of Beta-glucuronidase and snowdrop lectin genes in transgenic tobacco plants. *Journal of Experimental Botany* 45 (274), 623-631

Shibuya, N., Goldstein, I.J., van Damme, E.J.M. and Peumans, W.J. (1987) A mannose specific lectin from the snowdrop (*Galanthus nivalis*) bulb and its application to the purification of glycoproteins. Ixth International Symposium on Glycoconjugates, Lille France 1987, Abstract Fl

Shibuya, N., Goldstein, I.J. van Damme, E.J. and Peumans, W.I. (1988) Binding properties of a mannose-specific lectin from the snowdrop (*Galanthus nivalis*) bulb. *Journal of Biological Chemistry* 263, 728-34

Shivprasad, S., Pogue, G.P., Lewandowski, D.J., Hidalgo, J., Donson, J., Grill, L.K. and Dawson, W.O. (1999) Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. *Virology* 255(2), 312-23

Sijmons, P.C., Grundler, F.M.W., von Mende, N., Burrows, P.R. and Wyss, U. (1991) *Arabidopsis thaliana* as a new model host plant for plant-parasitic nematodes. *Plant Journal* 1, 245-254

Sijmons, P.C., Atkinson, H.J. and Wyss, U. (1994) Parasitic strategies of root nematodes and associated host cell responses. *Annual Review of Phytopathology* 32:235-59

Smith, K.M. (1972) A Textbook of Plant Virus Diseases. 3rd Edition Longman pp 515-519

Solis, D., Jimenez-Barbero, J., Kaltner, J.H., Siebert, H.C., von der Lieth, C.W. and Gabius, H.J. (2001) Towards defining the role of the gkycan as hardware in information storage and transfer: Basic principles, experimental approaches and recent progress. *Cells Tissues Organs* 168 (1-2), 5-23

Spiegel, Y. and McClure, M.A. (1991) Stage-specific differences in lectin binding to the surface of *Anguina tritici* and *Meloidogyne incognita. Journal of Nematology* 23 (2), 259-263

Spiegel, Y. and McClure, M.A., Kahne, I., Robertson, W.M. and Saloman, R. (1991) Wheat germ agglutinin bound to the outer cuticle of the seed gall nematodes *Anguina agrostis* and *Anguina tritici. Journal of Nematology* 23 (4), 451-456

Spiegel, Y., Mor, M. and Sharon, E. (1996) Attachment of *Pasteuria penetrans* endospores to the surface of *Meloidogyne javanica* second-stage juveniles. *Journal of Nematology* 28 (3), 328-334

Spiegel, Y., Kahne, I., Cohen, L. and Sharon, E. (1997) *Meloidogyne javanica* surface coat protiens: Characterization and lability. *Parasitology* 115 (5), 513-519

Spies, A.G. and Spence, K.D. (1985) Effect of a sublethal *Bacil-lus thuringensis* crystal endotoxin treatment on the larval midgut of a moth, *Manduca sexta:*a SEM study. *Tiss and Cell* 17, 379-394

Stinissen, H.M., Peumans, W.J., De Langhe, E. (1984) Abscisic acid promotes lectin biosynthesis in developing and germinating rice embryos. *Plant Cell. Rep.* 3:55-59

Stirling, G.R., Bird, A.F., and Cakuras, A.B. (1896) Attachment of *Pasteuria- penetrans* spores to the cuticles of root-knot nematodes. *Revue de Nematologie* 9 (3), 251-260

Sydenham, G.M., McSorley, R. and Dunn, R.A. (1997) Effects of temperature on resistance in *Phaseolus vulgaris* genotypes and on development of *Meloidogyne* species. *Journal of Nematology* 29 (1), 90-103

Subbotin, S. (1993) Evolution of modified food cells induced by sedentary nematodes in plant roots. *Russian Journal of Nematology* 1 (1), 17-26

Taylor, A.L. and Robertson, W. M. (1970) Location of *Tobacco Rattle Virus* in the nematode vector, *Trichodorus pachydermus* Seinhorst. *Journal of General Virology,* 6, 179-182

Taylor, A.L. and Sasser, J.N. (1978) Biology, identification and control of root-knot neamtodes (meloidogyne species) Cooperative Publications of the Department of Plant Pathology, North Carolina State University and U.S. Agency, International Development, Raleigh, pp 111

Thottappilly, G., Rossel, H.W., Reddy, D.V.R., Morales, F.J., Green, S.K. and Makkouk, K.M. (1990) Chapter 6. Vectors of virus and mycoplasma diseases: an overview. In Insect pests of food legumes. Ed S.R. Singh. John Wiley and Sons. Chichester pp 323 -342

Townsend R, Read RC, Turner MW, Klein NJ, Jack DL (2001) Differential recognition of obligate anaerobic bacteria by human mannose-binding lectin. *Clinical and Experimental Immunology,* 124 (2): 223-228

Triantaphyllou, A.C.H. (1979) Cytogenics of root-knot nematodes. *In* Root-knot nemtaodes (Meloidogyne species) Systematic, biology, control. Lamberti, F and Taylor, C.E. (Eds.) Academic Press London and New York pp 85- 109

Triantaphyllou, A.C.H. (1973) Environmenal sex differentiation of nematodes in relation to pest management. *Annual Review of Phtopathology*, 1, 41-462

Triantaphyllou, A.C.H. (1960) Sex determinatino in Meloidogyne incoognita Chitwood, 1949 and intersexuality in M. javanica (Treub, 1885) Chitwood, 1949. *Annals institute phytopathology Banaki* (N.S) 3, 12-31

Triplett, B.A, Quartrano, R.S. (1982) Timing, localization and control of wheat germ agglutinin synthesis in developing wheat embryos. *Developmental Biology*. 91:491-96

Trudgill, D.L., Kerry, B.R. and Phillips, M.S. (1992) Seminar: integrated control of nematodes (with particular reference to cyst and root-knot nematodes) *Nematologica* 38, 482-487

Trudgill DL (1967) The effect of environment on sex determination in *Heterodera rostochiensis. Nematologica* 13, 263-277

Tsien, R.Y. (1998) The Green fluorescent protein. *Annual Review of Biochemistry* 67:509-44

Tzortzakakis, E.A. and Trudgill, D.L. (1996) A thermal time based method for determining the fecundity of *Meloidogyne javanica* in relation to modelling its population dynamics. *Nematologica* 42, 1-7

Udhe, K., Koenig, R. and Lesemann, D.E. (1998) An onion isolate of *Tobacco Rattle Virus*: Reactivity with an antiserum to Hypochoeris mosaic virus, a putative furovirus, and molecular analsis of its RNA 2. *Archives of Virology* 143(6),1041-1053

Urwin, P.E., Atkinson, H.J, Waller, D.A. and McPherson M.J. (1995) Engineered oryzacystatin-I expressedin transgenic hairy roots confers resistancs to *Globodera pallida. Plant Journal* 8:121-131

Van Damme, E.J.M., Allen, A.K. and Peumans, W.J. (1987) Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (*Galanthus nivalis*) bulbs. *FEBS Lett* 215, 140-144

Van Damme, E.J.M., Allen, A.K. and Peumans, W.J. (1988) Related mannose-specific lectins from different species of the family Amaryllidaceae *Physiol Plant* 73, 52-57

Van Damme, E.J.M., and Peumans, W.J. (1988) Bioasynthesis of the snowdrop (Galanthus nivalis) lectin in ripening ovaries *Plant Physiology.* 86, 922-926

Van Damme, E.J.M., and Peumans, W.J. (1990a) Developmental changes and tissue distribution of lectin in *Galanthus nivalis* L. and *Narcissus* cv. Carlton. *Planta* 182, 605-609

Van Damme, E.J.M., and Peumans, W.J. (1990b) Isolectins in *Narcissus*: compexity, inter- and intraspecies diffrences and developmental control. *Physiol Plant* 79:1-6. Copenhagen

Van Damme, E.J.M., Kaku, H., Perini, F., Goldstein, I.J. Peeters, B., Yagi, F., Decock, B,. and Peumans, W.J. (1991a) Biosynthesis, primary structure and molecular cloning of snowdrop (*Galanthus nivalis* L.) lectin. *European Journal of Biochemistry* 202, 23-30

Van Damme, E.J.M., de Clercq, N., Classens, F., Hemschoote, K., Peeters, B., and Peumans, W.J. (1991b) Molecular cloning and characterization of mmultiple isoforms of the snowdrop (*Galanthus nivalis* L.) lectin. *Planta* 186: 35-43

Van Damme, E.J.M., Goldstein, I.J., and Peumans, W.J. (1991c) A comparative study of mannose-binding lectins from amaryllidaceae and alliaceae. *Phytochemistry* 30(2) 509-514

Van Damme, E.J.M., Goldstein, I.J., Vercammen, G., Vuylsteke, J. and Peumans, W.J. (1992) Lectins of members of the Amaryllidaceae are encoded by multigene famillies which show extensive homology. *Physiologia Plantarum* 86:245-252

Van Damme, E.J.M., and Peumans, W.J. (1995a) Lectins as plant defence proteins, In Lectins. Biomedical Perspectives. (Eds) A. Pusztai and S Bardocz. Taylor and Francis London pp 1 - 21

Van Damme, E.J.M., and Peumans, W.J. (1996) Progress in structural analysis of plant lectins through molecular cloning. Lectins: Biology, Biochemistry, Clinical Biochemistry – Volume 11:17-27

Van Damme, E.J.M., Peumans W.L., Pusztai, A. and Bardocz, S. (1998) Chapter 1 Plant lectins: a special class of plant proteins In: Handbook of plant lectins: Properties and biomedical applications John Wiley and Sons Chichester pp 3-30

Van Driessche E (1988) Structure and function of leguminosae lectins, in Advances in lectin Research, (Ed.) Franz, H vol. 1, pp.73-143, Berlin:VEB Verlag.

Van Emden HF and Bashford MA (1971) The performance of *Brevicoryne brassicae* and *Myzus persicae* in relation to plant age and leaf amino acids. *Entomogia Experimentalis Applicata* 14: 349-360

Vanparijs, J., Broekaert, W. F., Goldstein, I. J., Peumans, W. J. (1991) Hevein - an antifungal protein from rubber tree (*Hevea brasiliensis*) latex. *Planta,* 183(2): 258-24

Vassilakos, N., Vellios, E.K., Brown, E.C., Brown, D.J.F. and MacFarlane, S.A. (2001) Tobravirus 2b protein acts *in trans* to facilitate transmission by nematodes. *Virology* 279, 478-487

Vining, L.C. (1990) Functions of secondary metabolites. *Annual Review of Microbiology.* 44, 395-427

Visser, P.B., Brown, D.J.F., Brederode, F.T. and Bol, J.F. (1999) Nematode transmission of *Tobacco Rattle Virus* serves as a bottleneck to clear the virus population from defective RNAs. *Virology* 263, 155-165

Verwoed, T.C. Dekker, B.M.M. and Hoekema, A. (1989) A small scale procedure for the rapid isolation of plant RNAs. *Nucleic acids Research* 17(6), 2362

Wang, M., Boulter, D. and Gatehouse, J.A. (1992) A complete sequence of the rice sucrose synthase-1 (Rss-1) gene. *Pl. Molecular Biology.* 19, 881-883

Wang, D., MacFarlane, S.A. and Maule, J.A. (1997) Viral determinants of *Pea Early Browning Virus* seed transmission in Pea. *Virology* 234, 112-117

Washburn, J. O., Kirkpatrick, B. A., Haas-Stapleton, E. and Volkman, L.E. (1998) Evidence that the stilbene-derived optical brightener M2R enhances Autographa californica M nucleopolyhedrovirus infection of *Trichoplusia ni* and *Heliothis viresens* by preventing sloughing of infected midgut epithelial cells. *Biological Control,* 11(1): 58-69

Weiss, W.I. and Drickamer, K. (1996) Structural basis of lectin-carbohydrate recognition. *Annual Review of Biochemistry* 65, 441-73

White and Morris, A.K. and Morris, J.T. (1994) Nonhomologous RNA recombination in Tombusviruses: Generation and evolution of defective interfering RNAs by stepwise deletions. *Journal of Virology* 68 (1), 14-24

Wilkinson, T.L. and Douglas, A.E. (1998) Host cell allometry and regulation of the symbiosis between pea aphids, *Acyrthosiphon pisum,* and bacteria, *Buchnera. Journal of Insect Physiology* 44, 629-635

Williamson, V.M. (1999) Plant nematode resistance genes. *Current Opinion in Plant Biology* 2, 327-331

Wyss U. and Grundler, F.M.W. (1992) Feeding behaviour of sedentary plant parasitic nematodes. *Netherlands Journal of Plant Pathology* 98 Supplement 2, 165-173

Yang, N.S. and Russel, D. (1990) Maize sucrose synthase-1 promoter directs phloem cell-specific expression of *gus* gene in transgenic tobacco plants. *Proceedings of the National Academy of Science USA* **87**: 4144-4148

Zeigler-Graff, V., Guilford. P.J. and Baulcombes, D.C. (1991) *Tobacco Rattle Virus* RNA 1 29 K gene product potentiates viral movement and also affects symptom induction in tobacco. *Virology* 182(1), 145-55

APPENDIX I

Nematode bioassays - ANOVA analysis of root mass

Bioassay 1 NS differences between wet root weights. N= 0.388, F= 0333 and G=0.257

Bioassay 3 NS difference between root mass of each treatment. F=0.444, G=0.457, N=0.531 (sed 0.0522)

For combined bioassay 4 to 6

A

		Bioassays 4 to 6		
Bioassay	2	0.0606	13.04	
Bioassay.Block	27	0.0046	0.88	
Treatment	2	0.0088	1.67	0.197
Residual	56 (2)	0.00528		
Total	87 (2)			

B

	Treatment			
	Virus-free	TRV-fsGNA	TRV-GNA	s.e.d
SQRT mean root mass	0.4930	0.4599	0.4685	0.01875
(untransformed)	(0.2492)	(0.2171)	(0.2257)	
Replicates (n)	30	30	30	

Mean number of galls for generated after ANOVA analysis of gall data for bioassays 1, 2 and 3 (see Figure 5.?). Untransformed data shown in brackets

	Bioassay 1 Treatment				Bioassay 2 Treatment				Bioassay 3 Treatment			
	Virus-Free	TRV-fsGNA	TRV-GNA	s.e.d	Virus-Free	TRV-fsGNA	TRV-GNA	s.e.d min min-max max	Virus-free	TRV-fsGNA	TRV-GNA	s.e.d min min-max max
Mean	4.66	4.66	1.87	0.619	5.79	3.91	4.11	0.762	4.88	4.88	4.10	0.618X
number	(23.6)	(23.6)	(5)	(5.98)	(35.2)	(17)	(21.2)	0.660	(24.7)	(25.3)	(18.9)	0.507
of galls								0.539				0.364X
Rep	10	10	10		10	20	19		9	26	26	

Mean number of galls for generated after ANOVA analysis of gall data for bioassays 4 to 6 and 7 to 10 (Table B, see Figure 5.?). Untransformed data shown in brackets.

	Bioassays 4 to 6 Treatment				Bioassays 7 to 10 Treatment				
	Virus-free	TRV-fsGNA	TRV-GNA	s.e.d	virus-free	TRV-ΔGFP	TRV-fsGNA	TRV-GNA	s.e.d
SQRT Mean	4.94	4.26	3.83	0.398	5.273	4.816	5.241	4.487	0.2403
Number of galls	(27.5)	(19.2)	(17.1)		(29.02)	(24.31)	(28.61)	(21.66)	
Replicates (n)	30	30	30		40	40	40	40	

APPENDIX II

Statistical output for aphid response parameters follows (see Figure 6.2)

A) Analysis of development times of nymphs to adult stage - ANOVA with randomized complete blocking

source of variation	d.f. (m.v.)	s.s	m.s	v.r.	F pr
Bioassay stratum	3	69.112	23.037	14.57	
bioassay.Block stratum	36	56.914	1.581	1.20	
treatment	3	3.331	1.110	0.85	0.473
Residual	78 (39)	102.417	1.313		
Total	120 (39)	198.050			

B) Analysis of aphid survival and polymorphism – Pearson's Chi square test

	Aphid observations			
Treatment	Dead	Apterae	Alatae	Apterae + alatae
Virus-free	8	18	4	9
TRV-ΔFGP	6	20	1	13
TRV-fsGNA	7	23	4	6
TRV-GNA	5	29	2	4

Pearson chi-square value is 12 with 9 df. Probability level under null hypothesis is p= 0.214

C) Aphid fecundity (total number of nymphs) recorded after 7 days - linear Regression analysis

Summary of analysis of the fitted terms: Constant + bioassay + treat

source of variation	d.f.	s.s	m.s	v.r.	F pr
Regression	6	646	107.70	2.11	0.061
Residual	84	4293	51.10		
Total	90	4939	54.88	1.22	0.309
Change	-3	-187	62.19	1.22	0.309

A1 aphids survival during the nymph production - binomial distribution

Summary of analysis

	Mean number of surviving A1 aphids							
	Treatment							
Day	Virus-free	se	TRV-fsGNA	se	TRV-GNA	se	TRV-ΔGFP	se
2	0.999	0.0002	0.928	0.049	0.967	0.321	0.905	0.052
3	0.973	0.027	0.853	0.067	0.967	0.032	0.871	0.059
4	0.915	0.047	0.714	0.086	0.867	0.062	0.811	0.069
5	0.858	0.058	0.681	0.087	0.767	0.076	0.746	0.077
6	0.802	0.067	0.641	0.091	0.733	0.081	0.748	0.077
7	0.78	0.069	0.594	0.092	0.732	0.08	0657	0.083
8	0.581	0.111	0.358	0.126	0.663	0.118	0.647	0.111
9	0.589	0.106	0.278	0.114	0.595	0.117	0.529	0.112
10	0.559	0.106	0.314	0.127	0.479	0.117	0.488	0.110
11	0.491	0.113	0.245	0.123	0.409	0.120	0.425	0.114
12	0.477	0.117	0.085	0.082	0.272	0.113	0.302	0.11

Accumulation analysis of deviance

Day 2

Change	d.f.	deviance	mean deviance	Deviance ratio	approx. chi pr
+ bioassay	3	0.7671	0.2557	0.26	0.857
+ treatment	3	5.1794	1.7265	1.73	0.159
Residual	9	6.8074	0.7564		
Total	15	12.7539	0.8503		

Day 3

Change	d.f.	deviance	mean deviance	Deviance ratio	approx. chi pr
+ bioassay	3	2.045	0.682	0.68	0.563
+ treatment	3	5.247	1.749	1.75	0.155
Residual	9	11.768	1.308		
Total	15	19.060	1.271		

Day 4

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	3	0.6408	0.2136	0.21	0.887
+ treat	3	4.7723	1.5908	1.59	0.189
Residual	9	7.5035	0.8337		
Total	15	12.9167	0.8611		

Day 5

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	3	2.470	0.823	0.82	0.481
+ treat	3	3.002	1.001	1.00	0.391
Residual	9	10.027	1.114		
Total	15	15.499	1.033		

Day 6

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	3	0.682	0.227	0.23	0.877
+ treat	3	2.085	0.695	0.70	0.555
Residual	9	13.602	1.511		
Total	15	16.370	1.091		

Day 7

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	3	2.3793	0.7931	0.79	0.497
+ treat	3	2.9866	0.9955	1.00	0.394
Residual	9	7.9825	0.8869		
Total	15	13.3484	0.8899		

Day 8

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	1	3.994	3.994	3.99	0.046
+ treat	3	3.610	1.203	1.20	0.307
Residual	3	43891	1.630		
Total	7	12.495	1.785		

Day 9

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	1	7.251	7.251	7.25	0.007
+ treat	3	4.457	1.486	1.49	0.216
Residual	3	6.837	2.279		
Total	7	18.545	2.649		

Day 10

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	1	8.9749	8.9749	8.97	0.003
+ treat	3	1.8740	0.6247	0.62	0.599

Residual	3	1.3927	0.6963		
Total	7	12.2416	2.0403		

Day 11

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	1	4.8804	4.8804	4.88	0.027
+ treat	3	1.8466	0.6155	0.62	0.605
Residual	3	1.3470	0.6735		
Total	7	8.0739	1.3457		

Day 12

Change	d.f.	deviance	mean deviance	deviance ratio	approx. chi pr
+ bioassay	1	1.028	1.028	1.03	0.311
+ treat	3	5.284	1.761	1.76	0.152
Residual	3	2.625	1.313		
Total	7	8.937	1.490		

D) Analysis of A1 aphid weights recorded after 7 days - Linear regression analysis for the fitted terms: Constant + bioassay + treat

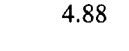
summary of analysis

source of variation	d.f.	s.s	m.s	v.r.	F pr
Regression	4	2.5×10^{-7}	6.303×10^{-8}	0.95	0.448
Residual	38	2.531×10^{-6}	6.66×10^{-8}		
Total	42	2.783×10^{-6}	6.33×10^{-8}		
Change	-3	-2.46×10^{-7}		1.23	0.311

E) Analysis of A1 aphid weights recorded after 12 days (bioassay 3 only) – Linear regression analysis for the fitted terms: Constant + bioassay + treat

summary of analysis

source of variation	d.f.	s.s	m.s	v.r.	F pr
Regression	3	$0.254E \times 10^{-6}$	$0.848E \times 10^{-8}$	1.45	0.274
Residual	13	$0.761E \times 10^{-6}$	$0.585E \times 10^{-8}$		
Total	16	$0.102E \times 10^{-5}$	$0.635E \times 10^{-8}$		



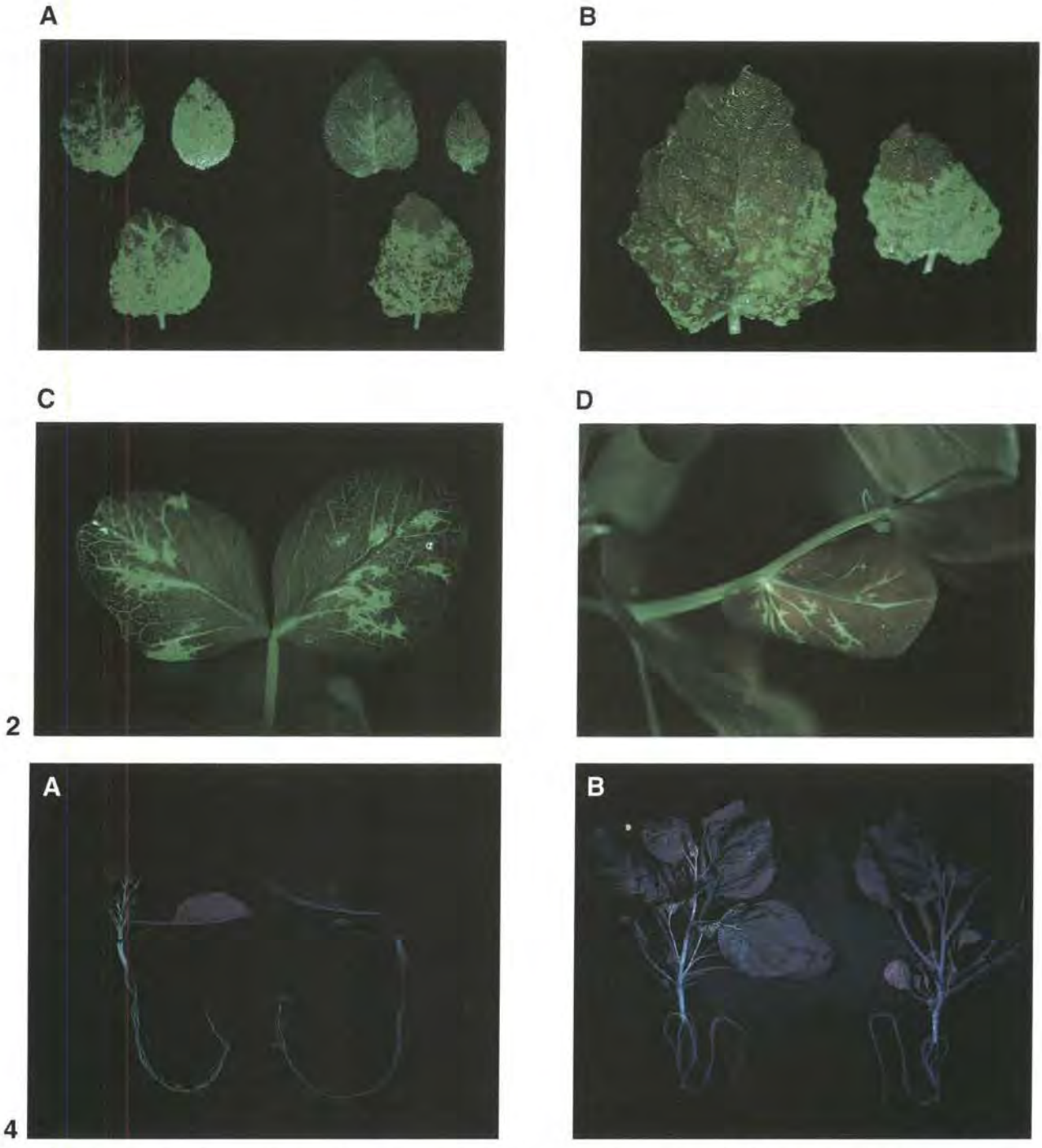


FIG. 2. Visualisation of GFP expression by UV illumination of tobravirus vector-infected plants. (A) Systemically infected leaves. Top row, *N. clevelandii*; bottom row, *N. benthamiana*. Left: TRV-GFP; right: PepRSV-GFP. (B) *N. benthamiana* systemically infected with PEBV-GFP. (C) Pea (*Pisum sativum* cv. Rondo) inoculated leaf infected with PEBV-GFP. (D) Systemic PEBV-GFP infection of pea.

FIG. 4. Comparison by UV illumination of TRV-GFP and PVX-GFP infection of *N. benthamiana*. (A) TRV-GFP at 6 dpi (left), mock-inoculated (right). (B) PVX-GFP at 9 dpi (left), mock-inoculated (right).

roots of infected plants, particularly to the region at the growing tip. We reasoned that significant benefits could be gained if tobraviruses could be used to express biologically active, nonviral proteins in plant roots. Such a system could have numerous applications including, for example, the testing of proteins involved in resistance to soil pests and pathogens, the bioremediation of contaminated soil and perhaps also the testing of candidate genes to manipulate the protein content/quality in root and tuber crops.

Examination of the roots of plants infected with each of the GFP-expressing tobraviruses revealed intense fluo-

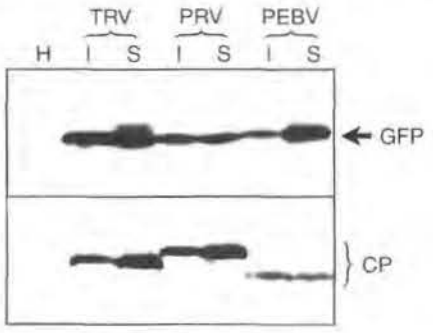


FIG. 3. Western blot of *N. benthamiana* infected with tobravirus vectors. Lanes were loaded with equal amounts of tissue extract. H, uninfected plant sample; I, inoculated leaf (sampled at 5 days postinoculation). S, systemically infected leaf, sampled at 7 dpi (TRV and PepRSV) and 8 dpi (PEBV). Top panel probed with GFP-specific antibodies. Bottom panel probed with a mixture of antibodies raised against TRV isolate PLB, PepRSV CAM, and PEBV SP5.

rescence particularly at root tips showing that the alterations made to RNA2 did not affect the pattern of virus movement (data not shown). The tobravirus vectors are not unique in their ability to express nonviral proteins in roots. For example tobacco etch potyvirus expressed high levels of GUS in tobacco lateral roots (Dolja *et al.*, 1992). Also, a TMV vector (30B-GFP; Shivprasad *et al.*, 1999) was found to produce large amounts of GFP in roots, although unlike TRV, the distribution of TMV in the roots was patchy (Popovich and MacFarlane, unpublished observations) and the virus did not invade the meristematic region (Oparka *et al.*, 1998).

Further experiments were carried out to compare the performance of the TRV expression vector with that of potato virus X (PVX), an alternative expression vector that is in the most widespread use (Chapman *et al.*, 1992; Baulcombe *et al.*, 1995).

Transcripts of TRV-GFP or PVX-GFP were inoculated to *N. benthamiana* plants, and extracts of systemically infected leaves exhibiting GFP-fluorescence were collected. These extracts were use to inoculate other *N. benthamiana* plants. Examination of whole plants by UV illumination showed that both viruses exhibited extensive GFP-fluorescence in infected leaves. In contrast, TRV produced significant GFP-fluorescence throughout the root system (Fig. 4A) whereas with PVX, root fluorescence was only apparent in the hypocotyl region, corresponding to an area of autofluorescence also found in the mock-inoculated plants (Figs. 4A and 4B). Root and leaf samples were taken from these plants for Western blotting when systemic GFP-fluorescence was at its strongest (TRV, 6 dpi; PVX, 9 dpi). In these experiments, TRV and PVX expressed GFP to similar levels in systemically infected leaves (Fig. 5). By contrast, however, TRV expressed 10- to 25-fold more GFP (as calculated from scanning densitometry of the blot) in the roots than did PVX.

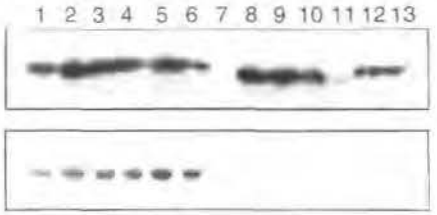


FIG. 5. Western blot of *N. benthamiana* infected with TRV-GFP and PVX-GFP. Lanes were loaded with equal amounts of tissue extract. Lanes 1–6, TRV-GFP infected plants at 6 dpi; lane 7, uninfected plant; lanes 8–13, PVX-GFP infected plants at 9 dpi. Top panel is extracts of leaves showing highest GFP-fluorescence, bottom panel is extracts of the complete root system excluding the hypocotyl. Blot probed with GFP-specific antibodies.

Stability of a nonviral insert cloned into TRV

A possible limitation of plant viruses as gene expression vectors is the tendency for nonviral sequences to be deleted during virus multiplication in the plant. Tobraviruses are known to be subject to recombination *in vivo* (Hernández *et al.*, 1996; MacFarlane, 1997). However, because the tobravirus RNA2 is dispensable for systemic infection of plants, it might be more amenable to the insertion of nonviral sequences than are some of the other, usually monopartite, plant viral vectors. In keeping with our proposal that TRV could be used to test anti-pest proteins, we decided to examine the stability in roots of a 12.5-kDa lectin (GNA) derived from snowdrop (*Galanthus nivalis* L.). This particular lectin has been shown to be inhibitory to insects (Powell *et al.*, 1995; Rao *et al.*, 1998) and might also, like some other lectins, be effective against nematodes (Marban-Mendoza *et al.*, 1987; Gupta and Sharma, 1993). Thus the TRV-GFP construct was modified by replacement of the GFP gene by the GNA gene. Plants were infected with TRV expressing GNA by inoculation to the leaves. Subsequently, at intervals, the roots of individual infected plants were collected and examined by Western blotting. GNA was expressed at uniform levels in different plants inoculated at the same time (data not shown). In these plants, GNA accumulated to ~10 μg per gram wet weight of root tissue. GNA was first detected in roots usually between 4 and 6 dpi and remained detectable for ≤24 dpi (the last time point to be

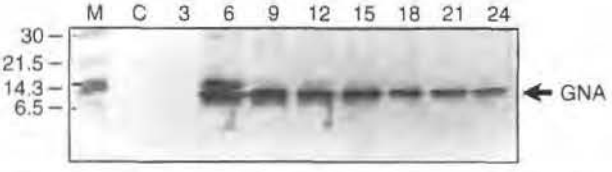


FIG. 6. Western blot of time course of GNA expressed from the TRV vector in roots of *N. benthamiana*. M denotes protein size markers (kDa). Equal amounts of root extract were loaded in each lane. C, uninfected plant sample. Other lanes are infected plants sampled at 3–24 days postinoculation. The blot was probed with GNA-specific antibodies.

FIG. 7. Western blot showing stability of GNA expression by TRV vector. Lanes 1–4 are equal amounts of root extracts of *N. benthamiana* infected with TRV-GNA. Lane 1 is first passage (7 dpi), lane 2 is second passage (14 dpi), lane 3 is third passage (11dpi), lane 4 is fourth passage (9 dpi). C, 50-ng purified GNA protein. H, root extract from a plant infected with TRV containing a frameshifted GNA gene. The blot was probed with GNA-specific antibodies.

analyzed) (Fig. 6). The longer term stability of the TRV-GNA virus construct was assessed by passaging extracts of infected roots to the leaves of other plants at intervals of between 7 and 14 days, with root samples being taken at each passage. In this experiment, root expression of GNA was maintained for four passages, at which time the experiment was terminated (Fig. 7). In addition, tissue printing of plants inoculated with TRV-GNA showed that GNA expression was widespread throughout the root system (Fig. 8).

Key features of tobravirus vectors

The tobraviruses have a number of features that make them attractive as gene expression vectors. The smaller viral RNA, RNA2, is nonessential for systemic infection of plants by the virus, which means that it can be extensively modified without affecting virus viability. The CP gene sgRNA promoters of these viruses are, to an extent, interchangeable, which allows the construction of relatively stable constructs containing additional promoters. This raises the possibility that constructs might be built that can express more than one nonviral protein. Tobraviruses, particularly TRV, have a wide host range, suggesting that they could be used as gene vectors in many plant species. Lastly, in contrast to many other plant viruses, tobraviruses are adapted for efficient movement into the root system. This property makes them particularly useful as delivery vectors for testing a wide variety of proteins that may be active in plant-soil/pathogen interactions.

MATERIALS AND METHODS

Clone construction

The TRV-GFP construct was derived from a full-length clone of RNA2 of TRV isolate PpK20 (pTR9598), which carries an additional *Apa*I restriction site downstream of the 2c gene at nucleotide 3470 (Mueller *et al.*, 1997). The 3′ part of the 2b gene and all of the 2c gene were removed by digestion with *Bst*EII (nucleotide 1636) and *Apa*I (nucleotide 3467), and replaced with a *Apa*LI–*Sma*I fragment containing the PEBV CP promoter [nucleotides 273–509, pFLA56 (MacFarlane and Brown, 1995)] linked to the GFP gene derived from plasmid pBAD-GFPcycle3 (Crameri *et al.*, 1996). The initiation codon of the GFP gene precisely replaced that of the viral CP gene. The cycle3 GFP gene contains a *Nhe*I site immediately after the initiation codon (ATGGCTAGC) and *Eco*RI and *Kpn*I sites downstream of the termination codon. Thus the GFP gene can be removed from TRV-GFP by digestion with *Nhe*I and *Eco*RI or *Kpn*I and replaced with other genes of choice. An additional vector (TRV-GFPc) includes a *Nco*I site at the GFP initiation codon (ccATGGCTAGC), allowing a more precise replacement of the GFP gene.

The PEBV-GFP construct was derived from pT72A56, a full-length cDNA clone of RNA2 of PEBV isolate TpA56 (MacFarlane *et al.*, 1996). The 2b and 2c genes were removed by digestion with *Bst*BI (nucleotide 1377) and *Eco*RI (nucleotide 2871), and replaced with a *Cla*I–*Eco*RI PCR fragment containing the TRV PpK20 CP promoter (nucleotides 346–556, pT72K20; Mueller *et al.*, 1997) linked to the GFP gene. As above, the GFP gene can be removed by digestion with *Nhe*I and *Eco*RI or *Kpn*I.

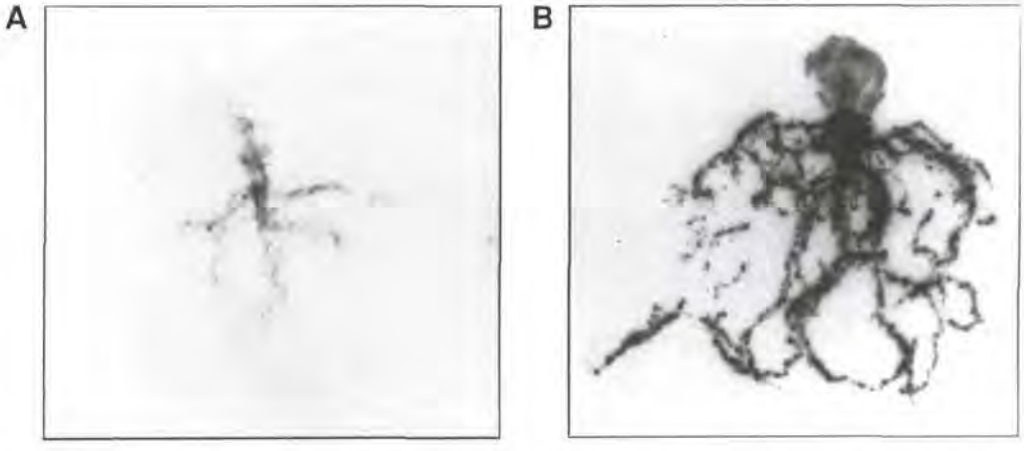


FIG. 8. Tissue print of *N. benthamiana* roots. (A) Mock-inoculated plant. (B) Plant infected with TRV expressing GNA, 21 days postinoculation. The prints were probed with antibodies raised against GNA.

The PepRSV-GFP construct was derived from a full-length clone (pT7Blue-PepRSV12) of RNA2 of PepRSV isolate CAM, which was obtained by long template-PCR (MacFarlane, 1996). A synthetic linker was used to introduce *Bsu*36I and *Bsi*WI sites at nucleotide 1251, downstream of the CP gene. A *Cel*II–*Kpn*I PEBV CP promoter-GFP fragment, derived from TRV-GFP, was introduced between the *Bsu*36I and *Bsi*WI sites. RNA2 of PepRSV isolate CAM carries at its 3′ end a partial copy of the RNA1-derived 16K gene. Thus in the PepRSV-GFP construct, this sequence is located 100 nucleotides downstream of the GFP gene.

The GFP gene was removed from TRV-GFP by digestion with *Nhe*I and *Kpn*I and replaced with the snowdrop lectin GNA gene (pGNA2; Gatehouse *et al.,* 1997) that had been PCR amplified to include a *Avr*II site at the second codon of the N-terminal signal sequence and a *Kpn*I site downstream of the termination codon.

Inoculation and analysis of plants

The TRV-GFP, TRV-GNA, and PEBV-GFP constructs were linearised at the 3′ terminus of the viral sequence by digestion with *Sma*I. PepRSV-GFP was linearised with *Spe*I, which cleaves the plasmid vector 10 bases downstream of the virus sequence. Transcript RNA was synthesised using T7 RNA polymerase as described before (MacFarlane *et al.,* 1996). Transcript RNA2 was mixed with RNA isolated from a plant infected only with RNA1 of each of the three viruses, a so-called NM-infection (Mueller *et al.,* 1997), and inoculated to *N. benthamiana* plants. Alternatively, infectious RNA1 transcripts can be synthesised from full-length cDNA clones of PEBV isolate SP5 (MacFarlane *et al.,* 1991) and TRV isolate PpK20 (S.MacFarlane, unpublished). The plants were examined at regular intervals for GFP fluorescence by illumination with long-wave UV light. Plants were photographed using Kodachrome 200 film with a HOYA G filter (for leaves) or without a filter (for whole plants). Extracts of transcript-inoculated plants were used to infect further plants. Leaf and root samples from infected plants were homogenised in 1 ml/g 1× PBS for analysis by Western blotting (Schmitt *et al.,* 1998). Tissue printing of roots was carried out as described before (Mansky *et al.,* 1990).

ACKNOWLEDGMENTS

We thank Walter Robertson, Vivian Blok, and Mark Phillips (Nematology Department, SCRI) for help and advice with the nematode bioassays. We also thank John and Angharad Gatehouse (Durham University) for providing us with the GNA plasmid clone, GNA2 protein, and GNA-specific antiserum. The GFP-specific antiserum and PVX-GFP were kindly provided by Simon Santa Cruz (Virology Department, SCRI). SCRI is grant-aided by the Scottish Office Environment Agriculture and Fisheries Department.

REFERENCES

Baulcombe, D.C., Chapman, S., and Santa Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for plant virus infections. *Plant J.* **7**, 1045–1053.

Bergh, S. T., Koziel, M. G., Huang, S. C., Thomas, R. A., Gilley, D. P., and Siegel, A. (1985). The nucleotide sequence of tobacco rattle virus RNA2 (CAM strain). *Nucleic Acids Res.* **13**, 8507–8518.

Brennan, F. R., Bellaby, T., Helliwell, S. M., Jones, T. D., Kamstrup, S., Dalsgaard, K., Flock, J.-I., and Hamilton, W. D. O. (1999). Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *J. Virol.* **73**, 930–938.

Chapman, S., Kavanagh, T., and Baulcombe, D. (1992). Potato virus X as a vector for gene expression in plants. *Plant J.* **2**, 549–557.

Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. C. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* **14**, 315–319.

Dolja, V. V., McBride, H. J., and Carrington, J. C. (1992). Tagging of plant potyvirus replication and movement by insertion of β-glucuronidase into the viral polyprotein. *Proc. Natl. Acad. Sci. USA* **89**, 10208–10212)

Dolja, V. V., Peremyslov, V. V., Keller, K. E., Martin, R. R., and Hong, J. (1998). Isolation and stability of histidine-tagged proteins produced in plants via potyvirus gene vectors. *Virology* **252**, 269–274.

Donson, J., Kearney, C. M., Hilf, M. E., and Dawson, W. O. (1991). Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. USA* **88**, 7204–7208.

Gatehouse, A. M. R., Davison, G. M., Newell, C. A., Merryweather, A., Hamilton, W. D. O., Burgess, E. P. J., Gilbert, R. J. C., and Gatehouse, J. A. (1997). Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: Growth room trials. *Mol. Breed.* **3**, 49–63.

Gupta, R., and Sharma, N. K. (1993). A study of the nematicidal activity of allicin—an active principle in garlic, *Allium sativum* L., against root-knot nematode, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949. *Int. J. Pest Manag.* **39**, 390–392.

Hammond-Kosack, K. E., Staskawicz, B. J., Jones, J. D. G., and Baulcombe, D. C. (1995). Functional expression of a fungal avirulence gene from a modified potato virus X genome. *Mol. Plant-Microbe Interact.* **8**, 181–185.

Harrison, B. D., and Robinson, D. J. (1986). Tobraviruses. *In* "The Plant Viruses" (M. H. V. van Regenmortel and H. Fraenkel-Conrat, Eds.), Vol. 2, pp. 339–369. Plenum Press, New York.

Hernández, C., Carette, J. E., Brown, D. J. F., and Bol, J. F. (1996). Serial passage of tobacco rattle virus under different selection conditions results in deletion of structural and non-structural genes in RNA2. *J. Virol.*, **70**, 4933–4940.

Hernández, C., Visser, P. B., Brown, D. J. F., and Bol, J. F. (1997). Transmission of tobacco rattle virus isolate PpK20 by its nematode vector requires one of the two non-structural genes in the viral RNA2. *J. Gen. Virol.* **78**, 465–467.

Joelson, T., Akerblom, L., Oxelfelt, P., Strandberg, B., Tomenius, K., and Morris, T. J. (1997). Presentation of a foreign peptide on the surface of tomato bushy stunt virus. *J. Gen. Virol.* **78**, 1213–1217.

Kumagai, M. H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. K. (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. USA* **92**, 1679–1683.

Kumagai, M. H., Turpen, T. H., Weinzettl, N., Della-Cioppa, G., Turpen, A. M., Donson, J., Hilf, M. E., Grantham, G. L., Dawson, W. O., Chow, T. P., Piatak, M., and Grill, L. K. (1993). Rapid, high-level expression of biologically active α-trichosanthin in transfected plants by an RNA viral vector. *Proc. Natl. Acad. Sci. USA* **90**, 427–430.

Lacomme, C., Smolenska, L., and Wilson, T. M. A. (1998). Genetic engineering and the expression of foreign peptides or proteins with plant virus-based vectors. *In* "Genetic Engineering" (J. K. Setlow, Ed.), Vol. 20, pp. 225–237. Plenum Press, New York.

MacCullock, L. A. (1991). "Chemotactic and electrotactic localisation of

plant roots by parasitic nematodes." Ph.D. thesis, University of Aberdeen, Aberdeen, Scotland.

MacFarlane, S. A. (1996). Rapid cloning of uncharacterised tobacco rattle virus isolates using long template (LT) PCR. *J. Virol. Methods* **56,** 91–98.

MacFarlane, S. A. (1997). Natural recombination among plant virus genomes: Evidence from tobraviruses. *Semin Virol.* **8,** 25–31.

MacFarlane, S. A., and Brown, D. J. F. (1995). Sequence comparison of RNA2 of nematode-transmissible and nematode non-transmissible isolates of pea early-browning virus suggests that the gene encoding the 29 kDa protein may be involved in nematode transmission. *J. Gen. Virol.* **76,** 1299–1304.

MacFarlane, S. A., Wallis, C. V., and Brown, D. J. F. (1996). Multiple genes involved in the nematode transmission of pea early browning virus. *Virology* **219,** 417–422.

MacFarlane, S. A., Wallis, C. V., Taylor, S. C., Goulden, M. G., Wood, K. R., and Davies, J. W. (1991). Construction and analysis of infectious transcripts synthesized from full-length cDNA clones of both genomic RNAs of pea early-browning virus. *Virology* **182,** 124–129.

Mansky, L. M., Andrews, R. E., Durand, D. P., Jr., and Hill, J. H. (1990). Plant virus location in leaf tissue by press blotting. *Plant Mol. Biol. Rep.* **8,** 13–17.

Marban-Mendoza, N., Jeyaprakash, A., Jansson, H. B., Damon Jr., R. A., and Zuckerman, B. M. (1987). Control of root-knot nematodes on tomato by lectin. *J. Nematol.* **19,** 331–335.

Mueller, A-M., Mooney, A. L., and MacFarlane, S. A. (1997). Replication of *in vitro* tobravirus recombinants shows that the specificity of template recognition is determined by 5′ non-coding but not 3′ non-coding sequences. *J. Gen. Virol.* **78,** 2085–2088.

Oparka, K. J., Boevink, P., Roberts, A. G., Blackman, L., Palukaitis, P., MacFarlane, S. A., and Santa Cruz, S. (1998). The ins and outs of systemic virus transport. "Workshop on Plasmodesmata and Transport of Plant Viruses and Plant Macromolecules." Instituto Juan March, Centre for International Meetings on Biology, Madrid, Spain.

Porta, C., Spall, V. E., Loveland, J., Johnson, J. E., Barker, P. J., and Lomonossoff, G. P. (1994). Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides. *Virology* **202,** 949–955.

Powell, K. S., Gatehouse, A. M. R., Hilder, V. A., and Gatehouse, J. A. (1995). Antifeedent effects of plant lectins and an enzyme on the adult stage of the rice brown planthopper *Nilaparvata lugens*. *Entomol. Exp. Appl.* **75,** 51–59.

Rao, K. V., Rathore, K., Hodges, T. K., Fu, X., Stoger, E., Sudhakar, D., Williams, S., Christou, P., Bharathi, M., Bown, D. P., Powell, K. S., Spence, J., Gatehouse, A. M. R., and Gatehouse, J. A. (1998). Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown plant hopper. *Plant J.* **15,** 469–477.

Sablowski, R. W. M., Baulcombe, D. C., and Bevan, M. (1995). Expression of a flower-specific Myb protein in leaf cells using a viral vector causes ectopic activation of a target promoter. *Proc. Natl. Acad. Sci. USA* **92,** 6901–6905.

Schmitt, C., Mueller, A.-M., Mooney, A., Brown, D. J. F., and MacFarlane, S. A. (1998). Immunological detection and mutational analysis of the RNA2-encoded nematode transmission proteins of pea early browning virus. *J. Gen. Virol.* **79,** 1281–1288.

Shivprasad, S., Pogue, G. P., Lewandowski, D. J., Hidalgo, J., Donson, J., Grill, L. K., and Dawson, W. O. (1999). Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. *Virology* **255,** 312–323.

Taylor, C. E., and Brown, D. J. F. (1997). "Nematode Vectors of Plant Viruses." CAB International, New York.

Virology 267, 29–35 (2000)
doi:10.1006/viro.1999.0098, available online at http://www.idealibrary.com on IDEAL®

Efficient Expression of Foreign Proteins in Roots from Tobravirus Vectors

Stuart A. MacFarlane[1] and Alexandra H. Popovich

Virology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

Received October 1, 1999; returned to author for revision November 8, 1999; accepted November 17, 1999

Viral vectors were constructed from infectious cDNA clones of each of the three tobraviruses, tobacco rattle virus (TRV), pea early-browning virus (PEBV), and pepper ringspot virus (PepRSV). RNA2 of each of the three viruses was modified to carry an additional coat protein subgenomic promoter and was used to express green fluorescent protein (GFP) when inoculated to plants. The tobravirus expression vectors have a wide host range and were able to express GFP in, for example, *Nicotiana* species, tomato, pea, arabidopsis, and sugar beet. The TRV vector was able to invade and express GFP very efficiently in roots, whereas the widely used PVX vector was not.

Key Words: plant virus vectors; root delivery; plant biotechnology; tobraviruses.

INTRODUCTION

Several recent studies have demonstrated the use of plant viruses as vehicles to introduce and express nonviral genes in plants (Donson *et al.*, 1991; Chapman *et al.*, 1992; Dolja *et al.*, 1998). Many plant viruses multiply to high levels in plants, leading to concomitantly high levels of nonviral gene expression. Virus delivery does not lead to permanent incorporation of the transgene into plants, nevertheless, depending on which virus is used, virus multiplication (and gene expression) can continue for long periods (weeks or months). Plant virus expression vectors have several other potential advantages over the more commonly used transgenic plant technology. Firstly, plant RNA viruses often are small in size (between 3000 and 10,000 nucleotides) making them easy to manipulate *in vitro*. Secondly, infection of plants with engineered virus leads to the immediate expression of the heterologous gene with no requirement for the lengthy and complex tissue culture procedures that are necessary when producing stably transformed plants. Different viruses have been described that can infect plants from most of the taxonomic groups. Some viruses in particular have very wide host ranges themselves. Thus it is likely that plant virus gene vectors can be developed that would allow foreign gene expression in whichever plant species was required. Several strategies have been employed in the development of plant virus expression vectors (Lacomme *et al.*, 1998). Often the foreign gene either is carried as a replacement for a nonessential virus gene or is inserted as an additional gene linked to a duplicated viral promoter. Other plant viruses have been adapted to express foreign sequences fused to one end of, or within, the virus coat protein gene, or as part of a polyprotein that is cleaved during maturation. These approaches have been used to express a variety of different nonviral proteins, e.g., marker proteins—GUS (Dolja *et al.*, 1992) and GFP (Baulcombe *et al.*, 1995), α-trichosanthin (ribosome inactivating protein; Kumagai *et al.*, 1993), phytoene synthase (metabolic pathway enzyme; Kumagai *et al.*, 1995), myb-like transcription factor (Sablowski *et al.*, 1995), avr9 (fungal elicitor of disease resistance; Hammond-Kosak *et al.*, 1995) and several antigenic peptides for use in vaccine development (Porta *et al.*, 1994; Joelson *et al.*, 1997; Brennan *et al.*, 1999).

Plant virus expression vector technology can be improved along several lines. New vectors are needed to expand the range of plants in which these systems can be used. Different viruses might be more tolerant of sequence manipulations and may be less prone to insert instability. New vectors might be developed with increased carrying capacity or with the ability to express more than one nonviral protein. In addition, vectors based on alternative viruses might have different tissue tropisms allowing selective foreign gene expression in specific parts of the plant.

The tobraviruses tobacco rattle virus (TRV), pea early-browning virus (PEBV), and pepper ringspot virus (PepRSV) have a number of properties that suggested that they might be developed as gene expression vectors. These viruses have two positive-sense, single-stranded genomic RNAs that are separately encapsidated in rod-shaped particles (Harrison and Robinson, 1986). RNA1 encodes the viral proteins that are responsible for replication and movement of the virus in plants. Indeed, RNA1 can cause infection (a so-called NM-infection) in plants in the complete absence of the second

[1] To whom reprint requests should be addressed. Fax: +44 1382 562426. E-mail: s.macfarlane@scri.sari.ac.uk.

0042-6822/00 $35.00

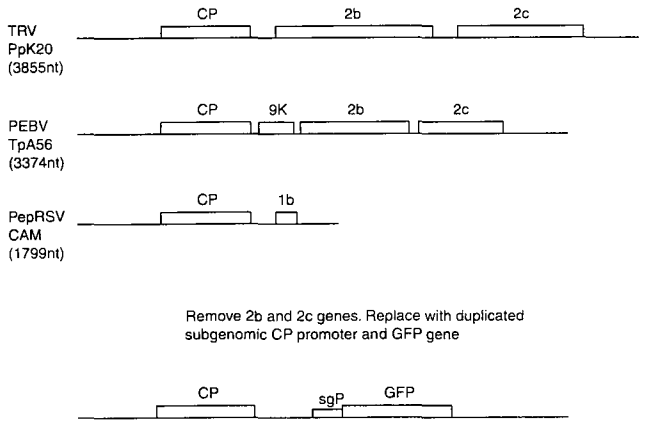


FIG. 1. Strategy for tobravirus vector construction. Gene organisation of RNA2 of isolates of TRV, PEBV, and PepRSV used in this study. CP, coat protein. The 2b and 2c genes encode nonstructural proteins involved in nematode transmission and are deleted from the vector constructs. A duplicated tobravirus CP gene subgenomic RNA promoter (sgP) is inserted upstream of the GFP gene. PepRSV RNA2 includes a partial copy of the RNA1-encoded 1b gene.

RNA (RNA2). RNA2 varies in size, according to virus isolate, and encodes the virus coat protein and sometimes one or more other, nonstructural proteins.

As RNA2 is inessential for infection of plants, we anticipated that it could be engineered to express nonviral genes. Previously, experiments using infectious cDNA clones of PEBV and TRV RNA2 showed that removal of the nonstructural 2b and 2c genes (Fig. 1) did not prevent movement to and replication of the viruses in systemic tissue (MacFarlane *et al.,* 1996; Hernandez *et al.,* 1997). Prior to the work described in this paper, modification of PepRSV had not been undertaken. The nonstructural genes on RNA2 have been shown to be necessary for transmission of tobraviruses by vector nematodes; thus constructs in which these genes are deleted should be effectively contained in the plants under test. In this paper we demonstrate the utility of TRV, PepRSV, and PEBV as gene expression vectors

RESULTS AND DISCUSSION

Analysis of tobravirus vectors expressing GFP

Clones of RNA2 of TRV isolate PpK20 and PEBV isolate TpA56 were modified to remove the nonstructural 2b and 2c genes (Fig. 1). RNA2 of PepRSV isolate CAM is a naturally occurring recombinant molecule lacking these two genes but carrying at its 3′ end a partial copy of the RNA1-derived 16K gene (Bergh *et al.,* 1985). To avoid homology-driven instability, a second coat protein (CP) gene subgenomic RNA (sgRNA) promoter, derived from a different tobravirus isolate was inserted downstream of each virus CP gene. Previous studies showed that TRV and PEBV can activate expression of each others CP sgRNA promoter (Mueller *et al.,* 1997); however, similar studies have not been carried out with PepRSV. The TRV and PepRSV expression vectors were engineered to contain a second, PEBV CP sgRNA promoter, whereas the PEBV vector carried a second, TRV promoter. In each virus vector, the duplicated promoter was used to drive expression of the green fluorescent protein (GFP) gene (Fig. 1).

When combined with RNA1 of the appropriate, homologous virus, transcripts of the three vector constructs caused a productive infection in inoculated plants. TRV-GFP infection produced fluorescent foci visible by eye in inoculated leaves of *Nicotiana benthamiana* by 3 days postinoculation (dpi) (Fig. 2A). GFP fluorescence often was apparent in upper, noninoculated leaves at 4 dpi. PEBV-GFP and PepRSV-GFP produced fluorescence in the inoculated leaves slightly more slowly (3–4 dpi) and also moved into noninoculated leaves slightly later (5–7 dpi) (Figs. 2A and 2B). A similar pattern of infection was seen when these viruses were inoculated to *N. clevelandii,* although systemic infection by PEBV-GFP was infrequent in this species. The intensity of GFP-fluorescence also differed between the viruses. TRV-GFP produced the brightest fluorescence, with PEBV-GFP being less bright and PepRSV-GFP producing the least fluorescence. The three engineered viruses were also examined for their ability to express GFP in plants other than *Nicotiana* species. TRV-GFP was able to infect the greatest number of plant species, moving locally in sugar beet and systemically in tomato, petunia, and arabidopsis. PepRSV-GFP and PEBV-GFP had reduced host ranges, although PepRSV-GFP infected tomato efficiently and PEBV-GFP expressed GFP in systemically infected pea leaves (Figs. 2C and 2D).

CP and GFP expression in inoculated and systemic infected leaves of *N. benthamiana* plants infected with the three viruses was examined by Western blotting (Fig. 3). TRV-GFP and PepRSV-GFP produced more CP in systemic rather than inoculated tissue, probably reflecting a more uniform infection of systemic tissue. TRV produced more GFP in systemic rather than inoculated tissue, whereas with PepRSV there was very little difference in the GFP content of these two tissues. For PEBV, systemic and inoculated tissues contained similar amounts of CP, whereas systemic tissue contained much more GFP than did inoculated tissue. The sensitivities of the virus CP-specific antibodies are not equivalent, thus they cannot be used to compare the amount of each virus in the samples; however, TRV produced the most GFP, both in inoculated and in systemic tissue.

Expression of GFP in roots

Tobraviruses are transmitted in the field by root-feeding nematodes from the genera *Trichodorus* and *Paratrichodorus* (Taylor and Brown, 1997). Consequently these viruses possess the ability to move efficiently within the