TRANSFORMATION OF *BRASSICA NAPUS* cv. WESTAR WITH THE BEET WESTERN YELLOWS VIRUS COAT PROTEIN GENE

By

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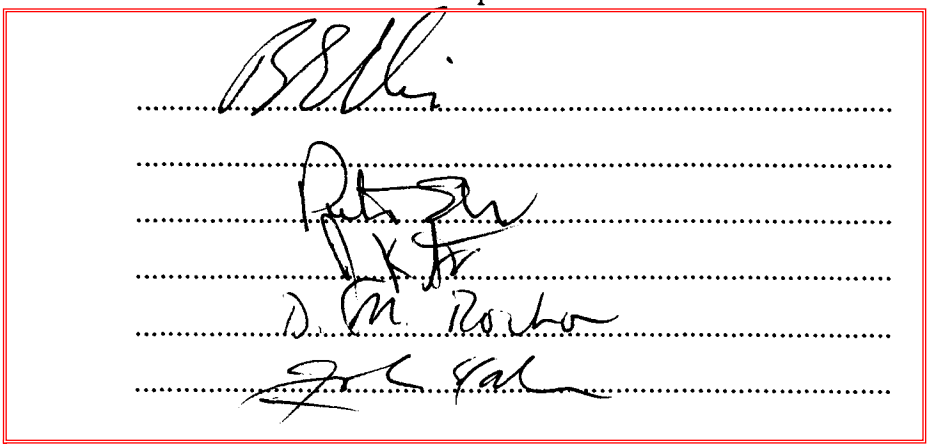

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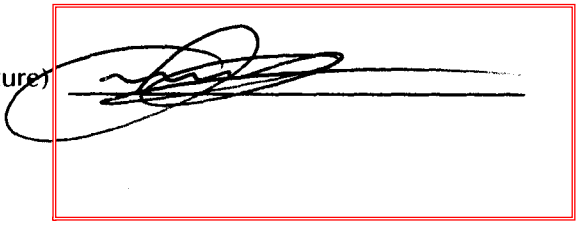
(Signature) 

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Abstract

Double stranded (ds) complementary DNA (cDNA) copies of the beet western yellows virus (BWYV) coat protein (CP) gene were synthesized from genomic BWYV RNA using reverse transcriptase (RT) followed by the polymerase chain reaction (PCR). The ds cDNA copy of the BWYV CP was then cloned into plasmid pRT103, a plant expression vector which contains the cauliflower mosaic virus (CaMV) 35*S* promoter and polyadenylation signal. The CP cDNA was inserted in the plus-sense orientation (clone BW102D) and in the anti-sense orientation (clone BW137D) relative to the CaMV 35*S* promoter. The CP coding regions of both pRT103 constructs were sequenced and found to correspond closely to those of previously published BWYV CP sequences. *In vitro* translation analysis in wheat germ extracts of synthetic transcripts of the cloned BWYV CP DNA produced only one major protein of *ca.* 22.5 kDa, the expected size for the BWYV coat protein. The CaMV 35*S* promoter-BWYV CP-polyadenylation cassette from clones BW102D and BW137D were then cloned separately into the *Hin*d III restriction enzyme site between the left and right borders of the T-DNA region and next to the *npt* II cassette of the two binary plasmids, pCGN1548 and pCGN1557 (Calgene). After mobilization of the four resulting binary plasmid constructs (pCGN154802, pCGN154837, pCGN155702 and pCGN155737) into *Agrobacterium tumefaciens* EHA101, the *A. tumefaciens* were used in the plant transformation procedure described by Moloney *et al.* (1989) utilizing cotyledonary

explants of *Brassica napus* cv. Westar. Thirty eight regenerated plants were recovered under the selection of the antibiotic, kanamycin. Southern blot analysis of *Hind* III digested plant DNA indicated that the BWYV CP gene was present in the genome of three transformed plants (154802-1, 154802-3 and 1557-7). PCR analysis of the same plant genomic DNA followed by Southern blot analysis of the PCR products indicated that several plants contained the BWYV CP gene integrated into the plant genome (plants 155702-1 to -8, -10 to -14, 1557-7 and 154802-1 to -5). RNA transcripts of either the BWYV CP gene or the co-transformed *npt* II gene could not be detected using Northern blot analysis of the RNA extracted from regenerated plants. However, germination of the R1 seeds from the regenerated plants on kanamycin indicated that the *npt* II gene product which confers kanamycin resistance was functional in many of the progeny of the regenerated plants. BWYV CP subunits could not be detected by enzyme-linked immunosorbent assay (ELISA) using a polyclonal antiserum which reacts with BWYV CP subunits. Preliminary evaluation of the progeny of two promising lines (154802-3 and 1557-7), demonstrated to have the BWYV CP gene integrated into the plant genome, did not reveal significant levels of resistance when challenged with the homologous virus using the aphid vector, *Myzus persicae* (Sulz.).

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List of Abbreviations

A_{260} - absorbance at 260 nm

AlMV - alfalfa mosaic virus

ATP (dATP) - adenosine triphosphate (deoxyadenosine triphosphate)

bp - base pair

BE - 40 mM boric acid / 1 mM EDTA (pH 8.2) buffer

BRL - Bethesda Research Laboratories

BSA - bovine serum albumin

BWYV - beet western yellows virus

BYDV - barley yellow dwarf virus

C - Celsius

ca. - about

CaMV - cauliflower mosaic virus

CAT - chloramphenicol acetyl transferase

cDNA - complementary DNA

Ci (uCi) - Curie (microCurie)

CIP - calf intestinal alkaline phosphatase

CMV - cucumber mosaic virus

CNV - cucumber necrosis virus

CTAB - hexadecyltrimethylammoniumbromide

CTP (dCTP) - cytidine triphosphate (deoxycytidine triphosphate)

cv. - cultivar

DAS-ELISA - double antibody sandwich ELISA

DNA - deoxyribonucleic acid

ds - double stranded

DTT - dithiothreitol

E (uE) - Einstein (microEinstein)

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

g (mg, ug, pg) - gram (milligram, microgram, picogram)

g - gravitational constant

GTP (dGTP) - guanosine triphosphate (deoxyguanosine triphosphate)

HCl - hydrochloric acid or hydrochloride

hr - hour

kb - kilobase

kbp - kilobase pair

KCl - potassium chloride

kDa - kiloDalton

L (mL, uL) - litre (millilitre, microlitre)

LB - Luria-Bertani

M_r - relative molecular weight

M (mM) - molar (millimolar)

m - metre

mas 5' - mannopine synthase promoter

mas 3' - mannopine synthase terminator

MeHgOH - methylmercuric hydroxide

M-MLV - Moloney murine leukaemia virus

$MgCl_2$ - magnesium chloride

$MgSO_4$ - magnesium sulfate

min - minute

mob - mobilization genes

mRNA - messenger RNA

NaCl - sodium chloride

NaOH - sodium hydroxide

NEN - New England Nuclear

*npt*II - neomycin phosphotransferase

nt - nucleotide

ORF - open reading frame

PAGE - polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

PEG - polyethylene glycol

PLRV - potato leafroll virus

poly (A) - polyadenylated

PVX - potato virus X

PVY - potato virus Y

RNA - ribonucleic acid

rpm - revolutions per minute

RT - reverse transcription

tra - transfer genes

s - second

SDS - sodium dodecyl sulfate

ss - single stranded

SSC - 20X SSC is 3M sodium chloride / 0.3M trisodium citrate (pH 7.5) buffer

T-DNA - transfer DNA

TE - 10 mM Tris-HCl (pH 7.4) / 1 mM EDTA buffer

TEV - tobacco etch virus

Ti - tumor-inducing

tml 3' - *tml* terminator from pTiA6

TMV - tobacco mosaic virus

TNE - 100 mM Tris-HCl (pH 7.5) / 100 mM NaCl / 10 mM EDTA

Tris - trishydroxymethylaminomethane

TSWV - tomato spotted wilt virus

TSV - tobacco streak virus

dTTP - deoxythymidine triphosphate

VPg - viral genome-linked protein

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Introduction

1.1 Research Objectives

Brassica napus, apart from being a major crop (over $1 billion annually in Canada), has proven to be amenable to a variety of methods which lead to the production of transgenic plants. The transformation methods include the use of *Agrobacterium* vectors (Radke *et al.*, 1988), microprojectile bombardment (Neuhaus *et al.*, 1987) and electroporation (Guerche *et al.*, 1987). Whole plants have been regenerated from a variety of tissues, such as stem explants (Stringham, 1979), leaf and root protoplasts (Newell *et al.*, 1984; Xu *et al.*, 1982) and microspores (Chuong *et al.*, 1988). Nevertheless, these methods are usually labour-intensive and the transformation efficiency low.

Brassica cultivars are known to be susceptible to beet western yellows virus (BWYV) disease (Gilligan *et al.*, 1980; Smith and Hinckles, 1985). Several preliminary studies suggest that the occurrence of BWYV in both *Brassica* cultivars and in a number of common weeds is significant (Ellis, 1992; Ellis and Stace-Smith, 1990; Hampton *et al.*, 1990; Thomas *et al.*, 1993) but the yield losses caused by BWYV have not been examined . Transgenic plants containing the coat protein gene of a virus have been shown to exhibit a delay or even complete inhibition of infection

when inoculated with the homologous virus. Such resistance was first demonstrated in tobacco for tobacco mosaic virus (TMV; Powell-Abel *et al.*, 1986) and since then for several different virus groups including the luteovirus group (Kawchuk *et al.*, 1990; van der Wilk *et al.*, 1990) with potato leafroll virus. The main objective of this study has been to attempt to confer genetically engineered resistance in *Brassica napus* cv. Westar to BWYV through the incorporation of the BWYV coat protein (CP) gene via *Agrobacterium tumefaciens* Ti plasmid mediated gene transfer. To this end, double stranded (ds) complementary DNA (cDNA) copies of the BWYV CP gene were synthesized from BWYV RNA (extracted from a sugarbeet isolate of BWYV) using reverse transcription (RT) followed by the polymerase chain reaction (PCR). The BWYV CP cDNA was cloned into the pRT103 plant expression vector (Töpfer *et al.*, 1987), under the regulatory control of the cauliflower mosaic virus (CaMV) 35*S* promoter and polyadenylation signal. The CaMV 35*S* promoter-BWYV CP gene-polyadenylation signal cassette was used to make binary Ti plasmid constructs in the pCGN15 series of vectors (McBride and Summerfelt, 1990). After mobilization of these binary plasmid constructs into *A.tumefaciens* EHA101 (Hood *et al.*, 1986), the *A. tumefaciens* were used in the plant transformation procedure described by Moloney *et al.* (1989) to transform *Brassica napus* cv. Westar. The regenerated plants were then evaluated.

1.2 Epiphytology of Beet Western Yellows Virus (BWYV)

Beet western yellows virus (BWYV), a member of the luteovirus group (Waterhouse *et al.*, 1988), is a very widespread and economically important plant virus. Unlike most luteoviruses, BWYV has a very wide host range including many dicotyledonous and at least one monocotyledonous plant species (Rochow and Duffus, 1981; Casper, 1988). Some commercially important crop plants susceptible to BWYV disease include sugarbeet, red beet, lettuce, broccoli, cauliflower, radish, turnip, flax and oilseed rape (Duffus, 1972; Gilligan *et al.*, 1980). BWYV occurs naturally in a number of common weed species and often in overwintering hosts of vector aphids (Ellis, 1992). Stunting and chlorosis (interveinal yellowing of the older or intermediate leaves) are typical symptoms of BWYV disease. BWYV is transmitted in the persistent circulative manner by several species of aphids, the most important of which is *Myzus persicae* (Sulz.). BWYV is not transmissible by sap inoculation. Furthermore, BWYV is phloem-limited and present in only very low concentrations in plant tissue extracts.

1.3 Genomic Organization of Beet Western Yellows Virus

The genome of BWYV consists of a monopartite single stranded (ss) plus-sense RNA, 5641 nucleotides in length (Veidt *et al.*, 1988; Falk *et al.*, 1989). The genome is encapsidated in an isometric particle *ca.* 26 nm in diameter. A genome

linked protein (VPg) is covalently attached to the 5'-end of the RNA and the 3'-end has no poly(A) tail. The nucleotide sequence of the genomic RNA of a lettuce isolate of BWYV has been determined (Veidt *et al.*, 1988). The BWYV genome is comprised of six large open reading frames (ORFs) (see Fig. 1.1). ORFs 1 and 2 are likely both expressed from genomic-length RNA and ORFs 4 and 5 from a single 3' co-terminal subgenomic RNA. Two other luteoviruses, barley yellow dwarf virus (BYDV) and potato leafroll virus (PLRV) employ a similar strategy for the expression of their corresponding ORFs (Dinesh-Kumar *et al.*, 1992; Tacke *et al.*, 1990; Lamb and Hay, 1990). ORF 1 encodes a polypeptide of 29 000 M_r (29 kDa), the function of which is not known. Overlapping extensively with ORF 1 but in a different reading frame is ORF 2 which encodes a polypeptide of 66 000 M_r (66 kDa). ORF 1 begins at the first AUG codon in the BWYV RNA sequence and ORF 2 at the second. Many viral RNAs are multicistronic but nevertheless are expressed in a monocistronic fashion; that is, ribosomes scan the RNA from the 5'-end until an AUG is encountered and only the 5' proximal ORF is translated. ORF 2 of BWYV is likely expressed by a 'leaky' scanning mechanism in which some of the ribosomes bypass the first AUG, which is in a sub-optimal context for translational initiation (Veidt *et al.*,1988), and initiate at the next AUG codon which is in a more favorable context (Kozak, 1989). The 3' terminus of ORF 2 shares a 474 nucleotide overlap with the beginning of ORF 3.

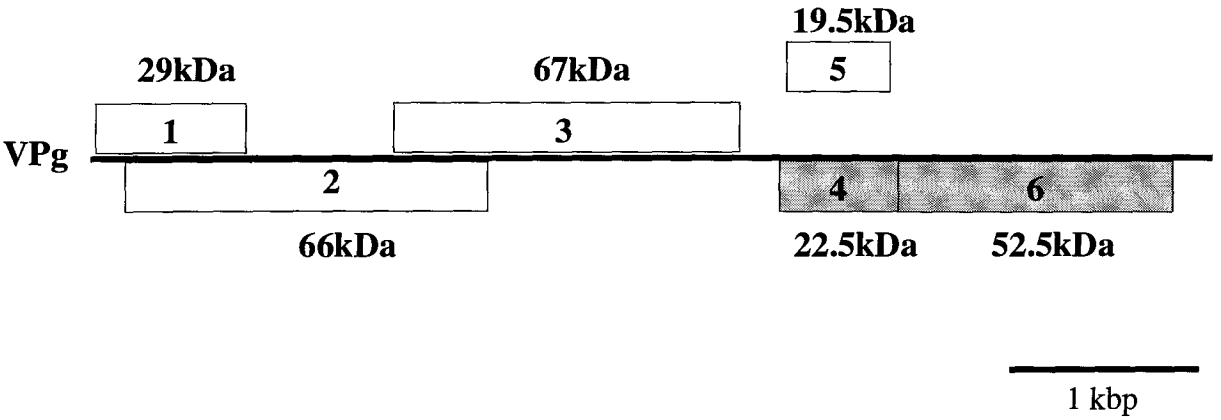


Fig. 1.1 Genomic organization of beet western yellows virus. The genome of BWYV consists of a monopartite single-stranded plus-sense RNA 5641 nucleotides in length. A viral genome-linked protein (VPg) is covalently attached to the 5' -end of the RNA and the 3' -end has no poly(A) tail. The BWYV genome is comprised of six large open reading frames (ORFs). Each ORF is represented by an open box with the predicted size of the protein product shown above or below the box. The shaded area corresponds to the BWYV coat protein and putative readthrough product.

Expression of ORF 3 involves a -1 translational frameshift somewhere in the region of overlap permitting translation as an ORF 2/3 frameshift fusion protein of approximately 115 kDa (Garcia *et al.*, 1993). Such a frameshift mechanism has been demonstrated for BYDV and PLRV (Miller *et al.*, 1988; Brault and Miller, 1992; Prüfer *et al.*, 1992; Mayo *et al.*, 1989; Kujawa *et al.*, 1993). The BWYV 67 kDa ORF (ORF 3) contains the amino acid sequence motif $GXXXTXXXNX_{18\text{-}50}GDD$, where X is any amino acid, present in nearly all known and putative viral RNA-dependent RNA polymerases (Goldbach, 1986). ORF 4 begins approximately 202 nucleotides after the stop codon for ORF 3 and encodes a polypeptide of 22 500 M_r (22.5 kDa). Comparison of the amino acid sequence coded for by this ORF with that of the coat protein ORF of BYDV has identified this BWYV ORF as the BWYV coat protein gene. Nested entirely within ORF 4, but in another open reading frame, is ORF 5, which encodes a polypeptide of 19 500 M_r (19.5 kDa). Comparison of the amino acid sequence coded for by ORF 4 with that of the similarly located 17 kDa ORF of BYDV suggests that the 19.5 kDa OEF may correspond to the VPg. Similar to ORF 2, ORF 5 is likely expressed by a 'leaky' scanning mechanism in which some ribosomes bypass the first AUG of ORF 4, which is in a suboptimal context for translational initiation, and initiate at the next AUG of ORF 5 which is in a more favorable context. Such a leaky scanning mechanism has been suggested for the corresponding 17 kDa ORF of PLRV (Tacke *et al.*, 1990). ORF 4 and 5 are expressed from a single 3' co-terminal subgenomic RNA (Falk *et al.*, 1989). ORF 6 immediately follows ORF 4 but ORF 4 terminates with an amber (UAG) termination

codon. It is possible that the UAG terminator of ORF 4 is read through by translational suppression of the ORF 4 amber termination codon to produce an ORF 4/6 fusion polypeptide of 74 000 M_r. Such translational suppression has been shown for the corresponding ORFs of BYDV and PLRV (Dinesh-Kumar *et al.*, 1992; Bahner *et al.*, 1990). A 5' region of the polypeptide encoded by ORF 6 appears to be an important determinant for aphid transmissibility (Bahner *et al.*, 1990). The corresponding ORF in PLRV (ORF 5) can encode a 53 kDa polypeptide which is probably derived from the rapid proteolysis of the 80 kDa/90 kDa proteins. The 80 kDa/90 kDa polypeptide resulting from the readthrough of the coat protein termination codon have been detected in extracts of partially purified particles; whereas only the 23 kDa coat protein subunit and the 53 kDa polypeptide could be detected in purified protein extracts (Bahner *et al.*, 1990). Indeed, a small number of protrusions have been detected on the surfaces of PLRV particles in favourably stained preparations (Harrison, 1984). Comparisons between aphid-transmissible isolates of PLRV and an isolate that is very poorly aphid-transmissible suggest that the determinant for aphid transmissibility is located in a region of the readthrough protein that is conserved at the amino acid sequence level among the three luteoviruses PLRV, BYDV and BWYV. The differences seen in aphid-transmissiblity between the PLRV isolates are a result of an alteration in this region as opposed to a deletion (Massalski and Harrison, 1987).

1.4 Genetically Engineered Resistance to Viral Infection

Hamilton (1980) first suggested that virus-induced resistance might be obtained by transferring a portion of a viral genome into a plant genome. The corresponding gene product(s) might provide protection against infection, either directly or indirectly, in a manner similar to that seen in cross-protection studies. If only a portion of the viral genome were used, the RNA transcript produced would not be infective. In addition, the protection provided by the transferred cDNA portion of the viral genome would be inherited from generation to generation.

Subsequent to this suggestion, several developments occurred which facilitated the introduction of foreign genes into plant genomes. Fraley (1983) reported the expression of bacterial genes in plants cells and Chilton *et al.* (1980) recognized the natural engineering ability of *A. tumefaciens*, to transfer the T-DNA (transfer DNA) portion of the tumour-inducing (Ti) plasmid into a wounded plant cell and have the T-DNA stably integrated into the plant genome. The first successful demonstration of genetically engineered resistance to a plant virus was reported by Powell-Abel *et al.* (1986) who found that transgenic tobacco plants expressing the coat protein gene from TMV displayed a significant delay in symptom development following inoculation with TMV. Since then, coat protein-mediated resistance has been demonstrated in several transgenic plants expressing the viral coat protein for a number of different viruses including alfalfa mosaic virus (AlMV; Van Dun *et al.*, 1987; Tumer *et al.*,

1987; Loesch-Fries *et al.*, 1987), cucumber mosaic virus (CMV; Cuozzo *et al.*, 1988), tobacco streak virus (TSV; Van Dun *et al.*, 1988), potato virus X (PVX; Hemenway *et al.*, 1988; Hoekema *et al.*, 1989), potato virus Y (PVY; Lawson *et al.*, 1990), potato leafroll virus (PLRV; Kawchuck *et al.*, 1990; van der Wilk *et al.*, 1991), tobacco etch virus (TEV; Lindbo and Dougherty, 1992) and tomato spotted wilt virus (TSWV; MacKenzie and Ellis, 1992; Goldbach *et al.*, 1992). While in some cases viral resistance was correlated with coat protein accumulation in the plant tissue (AlMV, TMV and CMV), in other cases, viral resistance was correlated with coat protein transcript accumulation (PVX, PVY, TEV, PLRV, TSWV). High levels of resistance to PLRV infection in transgenic potatoes in which the PLRV coat protein gene had been introduced, have been observed (Kawchuk *et al.*, 1990). Levels of PLRV coat protein transcripts were relatively high in the transgenic plants but coat protein represented less than 0.01% of the total leaf protein. Furthermore, resistance was found in transgenic plants containing either plus- or anti-sense transcripts of the PLRV coat protein gene.

It is clear that coat protein mediated resistance can reduce virus infection and disease development for a number of different host-virus systems but the molecular basis of resistance remains unknown and appears to differ between systems. Several mechanisms have been proposed for coat protein mediated resistance in transgenic plants including (reviewed by Beachy *et al.*, 1990): (1) interference with disassembly of the infecting virus or re-encapsidation of the infecting viral RNA, (2) interference

with the replication of the infecting viral RNA or (3) interference with the systemic spread of the infecting viral RNA.

1.5 *Agrobacterium tumefaciens* Mediated Gene Transfer into Plant Cells

A. tumefaciens is an opportunistic soilborne phytopathogen that can cause crown gall tumours in wounded gymnosperms and dicotyledonous angiosperms. This Gram-negative, rod-shaped bacterum is able to transfer a portion of its large (150-250 kb) Ti plasmid, the T-DNA, into the wounded plant cell and have the T-DNA stably integrated into the plant genome. The genes encoded on the T-DNA, while of bacterial origin, contain plant regulatory signals enabling their expression in infected plant cells. The expression of these genes has two consequences: (1) the synthesis of phytohormones necessary for the neoplastic transformation of the infected tissue to produce the characteristic tumorous gall, and (2) the synthesis of opines which serve as a carbon source for Agrobacteria harbouring a Ti plasmid and which induce the *tra* operon that allows the conjugal transfer of the Ti plasmid to other *Agrobacteria.* Garfinkel *et al.* (1981) observed that only two 25 bp border regions (imperfect repeats) of the T-DNA were essential for T-DNA transfer and that the remainder of the plasmid could be deleted and replaced with recombinant DNA. Using this approach, Bevan *et al.* (1983) and Herrera-Estrella *et al.* (1983) were the first to successfully introduce expressed foreign genes into plants. Herrera-Estrella *et al.* (1983) obtained expression of the octopine synthase and chloramphenicol acetyl transferase (CAT)

genes (regulated by the nopaline synthase promoter) in transgenic tumour tissue while Bevan *et al.* (1983) reported expression of neomycin phosphotransferase (*npt* II), a dominant selectable marker, in transgenic tumours. Since the Ti plasmids were too large (150-250 kb) for direct genetic manipulation, smaller vectors were designed that contained a selectable marker for introduction into *Agrobacterium*, with a selectable marker functional in plants between borders for T-DNA transfer to plants. These smaller vectors were more amenable to the manipulation of recombinant DNA in *Escherichia coli*. Garfinkel and Nester (1980) also discovered that the *vir* region of the Ti plasmid, containing the genes whose products are necessary for gene transfer, can act in *trans* on T-DNA carried on another plasmid. This observation led to development of two types of Ti-based vectors: cointegrate vectors and binary vectors. The cointegrate vectors have a region of homology between themselves and the accepting Ti plasmid. The cointegrate vectors contain pBR322 DNA (into which foreign genes for expression in plants can be cloned), the left and right borders of the T-DNA, a selectable marker for introduction into *Agrobacterium* (such as antibiotic resistance) and a selectable marker functional in plants (such as *npt* II for kanamycin resistance). These plasmids, having a ColE1 origin of replication, cannot be maintained in *Agrobacterium*. Instead, the cointegrate vectors are mobilized into *Agrobacterium* by triparental mating with a plasmid that supplies the *mob* and *tra* genes in *trans* (pRK2013; Ditta *et al.*, 1980), but only the Ti plasmid will be maintained in *Agrobacterium*; the antibiotic resistance gene from the cointegrate vector will allow for the selection of a single crossover event in which the cointegrate vector

becomes integrated between the borders of the Ti plasmid. Binary vectors, on the other hand, contain a broad host range origin of replication and a bacterial antibiotic resistance gene, for selection and maintenance in *Agrobacterium.* They also have T-DNA borders between which they typically carry a selectable marker expressed in plants and a polylinker region (containing multiple, unique restriction sites for cloning genes of interest). The pCGN15-derived plasmids are typical binary vectors (McBride and Summerfelt, 1990). The pCGN15 series of binary vectors contain the left and right borders of the T-DNA (from pTiA6), a ColE1 origin of replication and transfer origin (from pBR322) expressed in *E. coli*, the pRiHRI origin of replication from the *A. rhizogenes* root inducing plasmid for high stability in *Agrobacterium*, a *lac Z'* gene polylinker segment from pUC18 incorporated into the T-DNA (blue/white screening for recombinant cloned gene plasmids of interest in *E. coli*), a gentamycin bacterial resistance marker compatible for selection in both *E. coli* and disarmed *A. tumefaciens* strains and a choice between two types of chimeric *npt* II genes (plant selectable marker) under the regulatory control of either the CaMV 35*S* promoter and the *tml* terminator (*tml* 3', from pTiA6) or the mannopine synthase promoter (*mas* 5') and the mannopine synthase termination signals (*mas* 3'). These binary vectors can be mobilized into *Agrobacterium* by triparental mating or by direct electroporation, but are maintained independently of the disarmed Ti plasmid in *Agrobacterium.* Following triparental mating, the *Agrobacterium* harbouring the gene of interest within a modified T-DNA region can be used to infect plant tissue, which must then be regenerated into a whole plant (see Fig. 1.2).

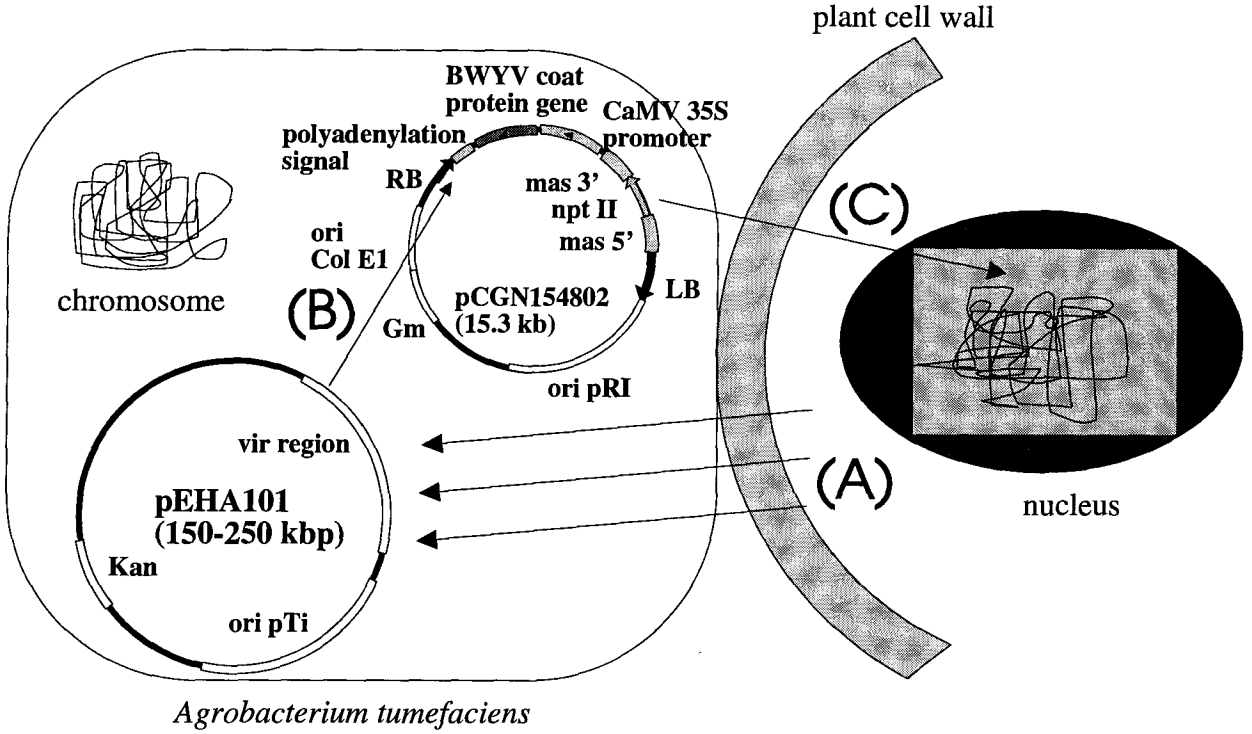


Fig. 1.2 *Agrobacterium tumefaciens* Ti plasmid mediated gene transfer. *Agrobacterium* move along a plant wound exudate concentration gradient and attach to the wounded plant cells. Wound exudate (A) stimulates the vir region of the disarmed Ti plasmid (pEHA101) to (B) act on the T-region and initiate (C) T- strand transfer and integration. The polarity of T-DNA transfer occurs from right border (RB) to the left border (LB). The example above shows the mobilized binary plasmid pCGN1548 in the *Agrobacterium tumefaciens* EHA101. The BWYV coat protein gene, under the control of the CaMV 35S promoter, was cloned in between the RB and LB of the Ti plasmid, pCGN1548. Kan= kanamycin resistance marker; Gm= gentamycin resistance marker; RB/LB= right/left border regions from pTiA6; ori pRI= origin of replication of the *A. rhizogenes* root inducing plasimd pRiHRI; mas5'/mas3'= mannopine synthase promoter /terminator signals from pTiA6; ori Col E1= origin of replication and transfer origin from pBR322; *npt* II= Tn5 neomycin phosphotransferase gene; ori pTi=origin of replication for the co-resident Ti plasmid. The shaded area on the pCGN154802 binary plasmid construct is the region that is transferred and integrated into the genome of the plant.

Materials and Methods

Procedures commonly used in molecular biology and which are used in this study are essentially as described by Sambrook *et al.* (1989). Restriction enzymes and DNA modifying enzymes were used according to the manufacturers' specifications unless otherwise stated.

2.1 BWYV Propagation and Purification

Beet western yellows virus was propagated in *Brassica napus* cv. Westar. Plants were inoculated by viruliferous *Myzus persicae* Sulz. in a growth chamber (21°C, 16 hr daylight/8 hr night, 60-80 $uEm^{-2}s^{-1}$ fluorescent and incandescent light) for a 2 to 3 day inoculation access period. The original BWYV, a sugarbeet isolate, was kindly provided by Peter Ellis, Agriculture Canada Vancouver Research Station. Plants were sprayed with Pirimor™ to kill the aphids and then placed in a greenhouse (18-23°C). Plants were harvested 4-6 weeks after inoculation. Virus-infected tissue was frozen in liquid nitrogen in an ice bucket, broken into coarse pieces with a wooden pestle and stored at -20°C.

The purification method used was a modification of the procedure described by D'Arcy *et al.* (1989). Approximately 250 g of frozen virus-infected tissue, was

ground until finely powdered in a Waring blender. Sodium phosphate buffer (0.1 M, pH 7.0) was added at a ratio of 2 mL per gram of tissue. Macerating enzyme (0.1% Ultrazym 100, Schweizerische Ferment AG, Basel, Switzerland), 2-mercaptoethanol (0.1% final concentration) and sodium azide (0.02% final concentration) were added and the slurry stirred until homogeneous. The slurry was left overnight at room temperature without stirring. The preparation was made to 1% Triton X-100 and clarified by vigorous stirring for 3 hr at room temperature followed by the addition of 1/6 volume chloroform:n-butanol (1:1) with continued vigorous stirring for 10 min at room temperature. After low speed centrifugation for 20 min at 8 500 rpm in a Sorvall GSA rotor, polyethylene glycol (PEG) 6000 and NaCl were added to the aqueous phase to a final concentration of 8% and 1%, respectively and stirred at 4°C overnight. The preparation was centrifuged as above and the pellets were resuspended in 0.1 M sodium phosphate buffer (pH 7.0) at a ratio of 10 mL per 50 g original tissue. The preparation was layered on a 5 mL pad of 20% sucrose in 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 60 000 rpm for 1 hr in a Beckman 70.1 rotor. The pellets were resuspended in 20 mL 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged for 20 min at 10 000 rpm in a Sorvall SS34 rotor. The supernatant was layered onto another 5 mL pad of 20% sucrose and centrifuged at 60 000 rpm for 1 hr in a Beckman 70.1 rotor. The pellets were resuspended in 0.5 mL 0.1 M sodium phosphate buffer (pH 7.0) with a glass rod, ground in a ground glass homogenizer and layered on top of a 10-40% linear sucrose density gradient prepared by the freeze-thaw method (Davis *et al.*, 1978). After centrifugation at

38 000 rpm for 1.5 hr in a Beckman SW41 rotor, the gradients were scanned at 254 nm using an ISCO density gradient fractionator. The fractions containing virus were collected and pelleted by centrifugation at 60 000 rpm for 1 hr in a Beckman 70.1 rotor. The final high speed pellet was resuspended with 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.0). The ultraviolet absorption spectrum (220 to 320 nm) of the preparation was recorded on a Hewlett-Packard Model 8451 A spectrophotometer. A yield of 100 ug of purified BWYV was typically obtained from 250 g of virus-infected tissue.

2.2 BWYV Virion RNA Extraction

Virion RNA was isolated from purified virus by extraction with phenol/chloroform in the presence of sodium dodecyl sulfate (SDS). To 300 uL of purified virus suspension (0.15 - 0.3 mg virus) was added 500 uL phenol (saturated with Tris-HCl buffer pH 8.0), 500 uL chloroform/octanol (24:1), 200 uL 0.5 M Tris-HCl (pH 8.9), 10 uL 100 mM EDTA (pH 8.0) and 50 uL 20% SDS. The mixture was vortexed and the aqueous and organic phases separated by centrifugation for 2 minutes in an Eppendorf centrifuge at 14, 000 x *g*. The aqueous phase was drawn off and the organic phase re-extracted with 500 uL sterile, deionized H_2O. The resulting aqueous phases were pooled and extracted first with an equal volume of phenol/chloroform/octanol (25:24:1) and then with an equal volume of chloroform/octanol (24:1). To the final aqueous phase was added 1/10 volume of 2 M

sodium acetate (pH 5.8) and 2 volumes of absolute ethanol. The RNA was precipitated at -70°C for 30 minutes and then centrifuged for 15 minutes at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in autoclaved deionized H_2O. The quality of the RNA was assessed by electrophoresis through denaturing agarose gels and was quantified spectrophotometrically (a 1 mg/mL solution of RNA has an A_{260} of 25). RNA samples were stored at -70°C.

2.3 Amplification of the BWYV Coat Protein Gene by Reverse Transcription and the Polymerase Chain Reaction

Double stranded (ds) complementary copies of the BWYV coat protein gene were synthesized from BWYV RNA using reverse transcriptase (RT) followed by the polymerase chain reaction (PCR), essentially as described by Sambrook *et al.* (1989). BWYV RNA (100 - 500 ng) was reverse transcribed in a 20 uL reaction volume in the presence of *Taq* DNA polymerase buffer (Promega) [50 mM KCl, 50 mM Tris-HCl (pH 9.0), 50 mM NaCl, 10 mM $MgCl_2$, 12.5 ug activated calf thymus DNA, 0.1 % Triton X-100]; 1.25 mM dATP, dCTP, dGTP, dTTP; 50 pmol of BWYV oligonucleotide primer #2 (5'-AGA AGG CCA TGG GCT AGG GC-3', complementary to a region just downstream from the coat protein gene at nucleotides 4108-4127 of the BWYV RNA); 20 units of RNase inhibitor (Promega); 2.5 mM $MgCl_2$ and 200 units of M-MLV reverse transcriptase (BRL). The reaction mixture was incubated at 37°C for 30 minutes and the M-MLV reverse transcriptase was

inactivated by heating the reaction mixture at 95°C for 5 minutes. To amplify the DNA generated by the reverse transcription, the reaction mixture was amended with 50 pmol of BWYV oligonucleotide primer #1 (5'-CGG CAC CAT GGA TAC GGT CGT GGG TAG G-3', identical to the sequence beginning at the coat protein initiator codon at nucleotides 3482-3503 of the BWYV RNA), 2.5 units of *Taq* DNA polymerase (Promega) and *Taq* DNA polymerase buffer to a final volume of 100 uL. The reaction mixture was overlaid with 100 uL of light mineral oil and the amplification reactions carried out in a thermocycler (EriComp). The first cycle of the PCR included denaturation at 94°C for 5 minutes, annealing at 55°C for 2 minutes and polymerization at 72°C for 2 minutes. This was followed with 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and polymerization at 72°C for 2 minutes. The final cycle of the PCR involved denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and polymerization at 72°C for 10 minutes. The mineral oil overlay was removed by the addition of 100 uL of chloroform/octanol (24:1) followed by brief centrifugation. The aqueous phase, which contained the amplified DNA, was drawn off and a sample was analysed by electrophoresis through a 1% agarose gel and visualized by staining with ethidium bromide. The amplified DNA from the PCR reaction was prepared for subcloning by digestion with *Nco* I restriction enzyme and the Double GeneClean™ manufacturer's procedure (Bio101 Inc.). DNA amplified in this manner was cloned into the *Nco* I site of the pRT103 plasmid. This strategy is described more thoroughly in the Results section 3.2.

2.4 DNA sequencing of the BWYV Coat Protein Gene

The dideoxynucleotide chain termination method of Sanger *et al.* (1977) was used to sequence two double stranded plasmid DNA templates (see Section 3.2.1) using a modified T7 DNA polymerase (Sequenase™, US Biochemicals). The following protocol was provided by US Biochemicals. Two micrograms of plasmid DNA and 10 ng of sequencing primer [BWYV oligonucleotide primer #1 or #2 or pRT103 oligonucleotide primer #1 (5'-CTT CCT CTA TAT AAG G-3') or #2 (5'-CTA CTC ACA CAT TAT TC-3')] were denatured in a 40 uL volume containing 200 mM NaOH and 0.4 mM EDTA by heating at 95°C for 5 minutes. The reactions were quickly cooled on ice and 1/10 volume of 2 M ammonium acetate and 2 volumes of absolute ethanol were added. The DNA was precipitated at -70°C for 30 minutes and then centrifuged for 15 minutes at 14, 000 x *g* in an Eppendorf microcentrifuge. The pellet was washed in 70% ethanol, dried and resuspended in 6 uL H_2O and 1.5 uL 5X Sequenase™ buffer [5X buffer is 200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$ and 250 mM NaCl]. The DNA was incubated at 37°C for 15 minutes to allow the primer to anneal to the denatured DNA template. To this solution was added 1 uL 100 mM DTT, 2 uL 1x labeling mix (1.5 uM each of dCTP, dGTP and dTTP), 2 to 5 uCi of α-[^{32}P]dATP (3000 Ci/mmol) and 3 units of Sequenase™ in a total volume of 13 uL. The reactions stood at room temperature for 2 to 5 minutes and 3.3 uL aliquots of this reaction mixture were added to tubes containing 2.5 uL 50 mM NaCl, 80 uM each of dGTP, dCTP, dTTP, dATP and 8 uM of either ddGTP, ddCTP, ddTTP or ddATP.

The reactions were incubated at 37°C for a further 20 minutes and to the reactions was added 4 uL of a mixture containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. The sequencing reactions were denatured in this mixture by heating at 95°C for 3 minutes before loading onto the sequencing gel. Sequencing reactions were electrophoresed through 6% polyacrylamide gels (using wedged spacers with 0.2 to 0.6 mm gradation) containing 6.7 M urea for 3-6 hours at constant power (48 watts). Following electrophoresis, the gels were transferred onto Whatman 3MM filter paper, dried under vacuum at 80°C for 1 hour and then exposed to Kodak X-Omat™ film overnight at room temperature. The sequences of the 5' and 3' ends (250 bp) of the BWYV coat protein cDNA were determined using the BW102D or BW137D clones and one of the primers, BWYV oligonucleotide #1 or #2 or pRT103 oligonucleotide #1 or #2. The sequence of the internal portion (200 bp) of the BWYV coat protein cDNA was determined using a deletion clone BW102DΔ*Sma* I and the pRT103 oligonucleotide primer #2.

2.5 *In Vitro* Translation Analysis of the BWYV Coat Protein Gene

2.5.1 *In Vitro* Transcription

Synthetic transcripts corresponding to the BWYV coat protein region were produced *in vitro* using the bacteriophage T3 RNA polymerase promoter and the pBluescriptII KS+ plasmid (Stratagene) containing the BWYV coat protein cDNA

insert from clone BW102D (see Section 3.3.1). The plasmid construct was linearized with *Sst* I restriction enzyme and used for run-off transcription. *In vitro* transcription reactions were carried out in the absence of cap analogue in a 50 uL volume containing 40 mM Tris-HCl (pH 8.0), 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 2.5 mM each of ATP, CTP, GTP and UTP, 10 mM DTT, 10 units RNasin, 50 units T3 RNA polymerase (BRL) and 2 ug linearized plasmid DNA. The reaction mixture was incubated at 37°C for 1 hour after which 0.2 units of DNase I was added and incubated for a further 15 minutes at 37°C. The reaction mixture was extracted with phenol/chloroform/octanol (25:24:1), the aqueous phase drawn off and 1/10 volume of 2M sodium acetate, pH 5.8 and 2 volumes of absolute ethanol added. The RNA was precipitated at -70°C for 1 hour, centrifuged for 15 minutes and the pellet resuspended in autoclaved deionized H_2O. The quality and amount of RNA transcript produced was determined by comparison with known quantities of cucumber necrosis virus RNA after agarose gel electrophoresis and ethidium bromide staining.

2.5.2 Translation *In Vitro* using Wheat Germ Extracts

Synthetic RNA transcripts, prepared as described above, were translated in wheat germ extracts (NEN) in the presence of [^{35}S]-methionine (NEN; specific activity 1200 Ci/mmol) according to the manufacturer's instructions. The 25 uL translation reaction, which included 50-100 ng synthetic RNA transcript, 50 uCi [^{35}S]-methionine, 12.5 uL wheat germ extract, 80 uM minus-methionine amino acid mixture and 75

mM potassium acetate, was carried out at 25°C for 1 hour. Translation products were analyzed following electrophoresis through sodium dodecyl sulfate containing polyacrylamide gels (SDS-PAGE, 15% gel, 0.75 mm thick) using the discontinuous Laemmli buffer system (Laemmli, 1970). Gels were then fixed in three changes of 30% methanol/10% acetic acid for 30 minutes each with continuous agitation according to the manufacturer's instructions. Gels were dried for 1 hour at 80°C under vacuum on Whatman 3MM paper and then fluorographed using Entensify (NEN) and exposed to X-ray film at -70°C overnight . The sizes of the translation products were estimated by comparison with the published sizes of the *in vitro* translation products of CNV (Rochon *et al.*, 1991).

2.6 Tri-Parental Mating Procedure

The triparental mating procedure was used to introduce the binary plasmid constructs (154802, 154837, 1548, 155702, 155737 and 1557) into *A. tumefaciens* EHA101 (Rogers *et al.*, 1986). The three bacteria involved in conjugation were (1) *E. coli* MM294 containing the binary plasmid constructs, (2) *E. coli* MM294 containing the mobilization plasmid, pRK2013 and (3) *A. tumefaciens* EHA101 containing a disarmed octopine type plasmid. In the triparental mating, the pRK2013 plasmid mobilized into the *E. coli* containing the binary plasmid construct, provided RK2 transfer proteins and the ColE1 *mob* protein which acts on the *bom* site of the binary plasmid and thereby mobilized the binary plasmid into *A. tumefaciens*. The

pCGN15-derived binary plasmids contained the ColE1 and pRiHRI origins for replication in both *E. coli* and *A. tumefaciens*, respectively. Overnight cultures of the *E. coli* and *A. tumefaciens* were started: *E. coli* containing the binary plasmid constructs of interest were grown in 2 mL of Luria-Bertani (LB) broth plus 10 ug/mL gentamycin, while the *E. coli*/pRK2013 and *A. tumefaciens* were grown in 10 mL LB plus 50 ug/mL kanamycin (the *Agrobacterium* was grown at 28°C to avoid loss of the Ti plasmid). One millilitre (mL) from each of the cultures was mixed together in a sterile tube, centrifuged and the pellet resuspended in 2 mL 10 mM $MgSO_4$. The mixture was filtered through a syringe filter (Nalgene™ 25mm, 0.45 um), the filter disc removed from the casing and transferred sterilely onto a fresh, nondried LB agar plate. The plate was incubated at 28°C overnight and the following day, the filter disc was removed aseptically and placed into a sterile tube containing 2 mL 10 mM $MgSO_4$. The tube was vortexed to remove cells from the filter disc and a 0.1 mL aliquot of the suspension was spread onto a freshly prepared LB agar selection plate containing 50 ug/mL gentamycin and 100 ug/mL kanamycin. The plates were incubated at 28°C for 3-4 days after which several hundred colonies appeared depending on the efficiency of the mating. Several colonies were inoculated into LB containing gentamycin and kanamycin and grown at 28°C for 16-24 hours. DNA from the *Agrobacterium* was extracted by the small nucleic acid preparation procedure (An *et al.*, 1988) and analyzed by agarose gel electrophoresis followed by ethidium bromide staining and by Southern blot analysis using a random primed, [^{32}P]-labeled cDNA BWYV coat protein probe (see Section 3.5). Glycerated bacterial cultures,

consisting of 0.15 mL sterile glycerol and 0.85 mL of the overnight culture, were stored at -70°C. *Agrobacterium* cells were also streaked onto LB plates containing gentamycin and kanamycin and stored at 4°C for 2 weeks; colonies were restreaked onto fresh plates every 2 weeks.

2.7 Transformation and Regeneration of *Brassica napus* cv. Westar

The plant transformation procedure used was a modification of the procedure described by Moloney *et al.* (1989) for transformation of *Brassica napus* cv. Westar utilizing cotyledonary explants. Four days after germination of seeds [on Murashige-Skoog minimal organics medium (MS) with 3% sucrose and 0.7% phytagar, pH 5.8; 24°C in a 16 hours light/8 hours dark photoperiod, 60-80 $uEm^{-2}s^{-1}$], cotyledons were excised and immersed into a bacterial suspension of *A. tumefaciens* EHA101 containing the binary plasmid of interest. The cotyledonary explants were co-cultivated with the *A. tumefaciens* for 72 hours (on MS medium, 3% sucrose and 0.7% phytagar, pH 5.8 enriched with 20 uM benzyladenine). The cut ends of *B. napus* cotyledons have been reported to be highly susceptible to *Agrobacterium*-mediated gene transfer and also to display very high regeneration rates (Moloney *et al.*, 1989). After 72 hours, the cotyledons were set out on regeneration medium (MS medium, 3% sucrose and 0.7% phytagar, pH 5.8 enriched with 20 uM benzyladenine) supplemented with selective antibiotics (15 mg/L kanamycin and 500 mg/L carbenicillin). Shoots appeared on the explants after 3-5 weeks; some shoots that were not transformed with

the *npt* II gene appeared bleached by the fourth week of culture on kanamycin. The shoots which remained green were sub-cultured first onto shoot elongation medium (regeneration medium without benzyladenine) for 4-6 weeks and then onto 'rooting' medium (MS medium, 3% sucrose, 4 mg/L indole butyric acid, 0.7% phytagar and 500 mg/L carbenicillin) for 4-6 weeks. No kanamycin was used during this stage since it was found that more rapid root establishment occurred without the selection agent. Plantlets were transferred to potting mix and placed in a misting chamber for 2-3 weeks after which the plants were transferred to the greenhouse and allowed to flower and set seed.

2.8 DNA Extraction from Plants using the CTAB Method

Plant genomic DNA was extracted from the regenerated *B. napus* cv. Westar by the procedure that utilizes hexadecyltrimethylammonium bromide (CTAB) as previously described by Doyle *et al.* (1990). One gram of fresh, leaf tissue was ground in liquid nitrogen in a chilled mortar and pestle. The powder was gently mixed into preheated (60°C) CTAB isolation buffer [2% (w/v) CTAB (Sigma), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0)] in a 30 mL centrifuge tube. The sample was incubated at 60°C for 30 minutes with occasional gentle swirling and afterwards, extracted with chloroform/isoamyl alcohol (24:1;v/v). After centrifugation at 1600 x *g* in a HB-4 rotor at room temperature, the aqueous phase was drawn off with a wide-bore pipet and 2/3 volume of cold

isopropanol was added to precipitate the nucleic acids. The sample was left at room temperature overnight. The sample was centrifuged at 500 x *g* in a SS34 rotor for 2 minutes, the supernatant poured off and 20 mL of wash buffer [76% (v/v) ethanol, 10 mM ammonium acetate] added. After 20 minutes of washing, the sample was centrifuged at 1600 x *g* for 10 min in a SS34 rotor and the pellet resuspended in 1 mL TE [10 mM Tris-HCl (pH 7.4), 1 mM EDTA]. RNase A was added to a final concentration of 10 ug/mL and incubated at 37°C for 30 minutes. The sample was diluted with 2 volumes of TE and the DNA precipitated with 7.5 M ammonium acetate (pH 7.7) added to a final concentration of 2.5 M and 2 volumes of cold ethanol. After centrifugation at 10,000 x *g* for 10 min in a SS34 rotor, the pellet was air dried and resuspended in 100 uL of TE. The final DNA sample was highly viscous. The yields, determined spectrophotometrically, ranged from 100 ug to 800 ug genomic DNA per two grams of tissue.

2.9 Synthesis of Random-Primed cDNA Probes

Random primed, [^{32}P]-labeled probes were prepared with the Random Primers DNA Labeling System (BRL) according to the manufacturer's instructions. Twenty-five nanograms of gel purified ds cDNA, which corresponded to the *Nco* I fragment of clone BW102D containing the BWYV coat protein gene (essentially the entire BWYV coat protein gene), was used as template. Specific activity of the probes was approximately 1.5 x 10^9 cpm/ug DNA.

2.10 Southern Blot Analysis

Plant genomic DNA samples (50 - 150 ug) were digested with *Hin*d III or *Eco* R1 restriction enzyme (2.5 - 5 times recommended units) at 37°C for 1.5 - 2.0 hours. The DNA samples were electrophoresed through a 1% agarose gel and stained with ethidium bromide. The gel was photographed under UV light (320 nm) and molecular size markers (Lambda DNA digested with *Eco* RI/*Hin*d III) referenced to a fluorescent ruler. The DNA was blotted onto Zeta-probe GT membrane (BioRad) under alkaline conditions (0.4 M NaOH) at room temperature for 8-16 hours according to the manufacturer's instructions. The following hybridization method was also according to the manufacturer's instructions (BioRad; Reed and Mann, 1985). The membranes were prehybridized in hybridization buffer (7% SDS, 0.25 M Na_2HPO_4, pH 7.2) at 65°C for 0.5 to 2 hours. Hybridization with random primed, [^{32}P]-labeled probe, corresponding to the BWYV coat protein gene or *npt* II gene, was carried out in a Hybaid oven (BioCan Scientific) for 16-24 hours at 65°C using approximately 1.9 x 10^6 cpm of probe (25 ng) per mililiter of hybridization buffer. After hybridization, the membranes were washed successively in 5% SDS, 20 mM Na_2HPO_4 (pH 7.2) and 1% SDS, 20 mM Na_2HPO_4 (pH 7.2). All washes were at 65°C for 30 minutes each. Excess moisture was removed from the membranes and they were wrapped in clear, plastic wrap. The membranes were exposed to X-ray film (X-Omat K, Kodak) at -70°C for 6 hours-7 days, depending on the experiment, with the aid of two

Lightening Plus intensifying screens (DuPont).

2.11 Leaf RNA extraction

Approximately 1-2 g leaf material was ground to a fine powder in liquid nitrogen, mixed vigorously with 3-5 volumes 10x TNE (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA), an equal volume of phenol/chloroform/octanol (25:24:1) containing 0.1% SDS and 5% 2-mercaptoethanol and centrifuged at 8 000 rpm for 5 minutes at 4°C (SS34 rotor). The aqueous phase was collected, re-extracted with an equal volume of phenol/chloroform/octanol (25:24:1), and then extracted with chloroform/octanol (24:1). The aqueous phase was combined with 1/10 volume of 2 M sodium acetate (pH 5.8) and 2 volumes of absolute ethanol, precipitated at -70°C for 1 hour and then centrifuged as above. The pellet was resuspended in 3 mL of TE, to which 1 mL 8 M LiCl was added. Samples were left on ice for 1-3 hours, then centrifuged at 8 000 rpm for 10 minutes at 4°C (SS34 rotor). The pellet, which contained high molecular weight single stranded RNA, was resuspended in 375 uL water, then re-precipitated with 1/10 volume 2 M sodium acetate (pH 5.8) and 2 volumes absolute ethanol. Samples were placed at -70°C for 1 hour, then centrifuged for 10 min at 4°C. Pellets were washed with 70% ethanol and the pellet resuspended in 50 uL of sterile, deionized water. Nucleic acid concentration was determined spectrophotometrically (one A_{260} unit of RNA= 40 ug/mL).

2.12 Northern blot analysis

Plant RNA , obtained as described above, was denatured with either 5 mM methylmercuric hydroxide (MeHgOH) and electrophoresed through 1% agarose gels prepared with BE buffer (40 mM boric acid, 1mM EDTA, pH 8.2) or with glyoxal and dimethyl sulfoxide and electrophoresed through 1% agarose gels prepared with 0.01 M sodium phosphate buffer, pH 7.0 (Sambrook *et al.*, 1989) . After electrophoresis, RNA was blotted onto Zeta-probe GT membranes (BioRad) under alkaline conditions (10 mM NaOH) at room temperature for 6-16 hours. The membranes were prehybridized in hybridization buffer (50% formamide, 7% SDS, 0.25 M NaCl, 0.12 M Na_2HPO_4, pH 7.2) for 1 hour at 42°C. Hybridization with a random primed, [^{32}P]-labeled probe, corresponding to the BWYV coat protein gene or the *npt* II gene, was carried out at 42°C for 6-16 hours. After hybridization, the membranes were washed successively in 2X SSC/0.1% SDS (20 X SSC is 3 M NaCl, 0.3 M trisodium citrate), 0.5X SSC/0.1% SDS and finally, in 0.1X SSC/0.1% SDS. All washes were conducted at 60°C for 15 min each. The membranes were wrapped in clear, plastic wrap and exposed to X-ray film at -70°C for 4 hours to 14 days with the aid of two intensifying screens (DuPont).

2.13 Production of Monoclonal Antibodies and Polyclonal Antisera

The production of monoclonal antibodies and polyclonal antisera which react against BWYV coat protein subunits or disrupted BWYV particles is essentially the same as that described for the production of monoclonal antibodies and polyclonal antisera which react against intact BWYV particles (Ellis and Wieczorek, 1992). For the production of monoclonal antibodies, BALB/c mice were immunized with at least three injections of virus (50 ug virus each injection). Prior to injection, the purified virus sample was treated in 1% SDS, heated at 95°C for 5 minutes, and dialyzed against 0.05 M phosphate buffer, pH 7.0. The first injection was 50 ug of purified disrupted virus particles emulsified with Freund's incomplete adjuvant and given subcutaneously. The second and third injections consisted of 50 ug each of purified disrupted virus particles in 0.05 M phosphate buffer given intraperitoneally. The injections were given at 3-4 week intervals. Three days after the third injection, the mice were sacrificed and the spleens harvested for use in the fusion protocol for hybridoma production. The hybridomas were screened for anti-BWYV (disrupted particles) antibody production by an indirect enzyme-linked immunosorbent assay (ELISA). Similarly, five purified BWYV preparations (disrupted by treatment in 1% SDS and heating at 95°C for 5 minutes followed by dialysis against 0.05 M phosphate buffer) were used to immunize a young, New Zealand White rabbit. The first injection, 0.1-0.5 mg of purified virus was disrupted and the subunits emulsified with an equal volume of Freund's complete adjuvant, was administered intramuscularly in a

hind leg. The subsequent injections of purified disrupted virus particles emulsified with Freund's incomplete adjuvant were given at 3-4 week intervals or longer, when purified virus samples were limited. IgG was purified from a test bleed after the fourth injection. The antiserum was evaluated in a double antibody sandwich-ELISA (DAS-ELISA) for reactivity with disrupted BWYV particles (treated as above or with carbonate buffer, pH 9.2).

2.14 Evaluation of Existing Monoclonal Antibodies in Western Blot and Dot Blot Analysis

Several monoclonal antibodies (510H 1gG2a α BWYV, 43BC IgM α BWYV & PLRV, 4G12 IgM α BWYV and 26BE IgG1 α PLRV) and polyclonal antisera (rabbit α BWYV IgG and rabbit α PLRV IgG), kindly provided by Peter Ellis (Agriculture Canada Research Station), were evaluated for reactivity with disrupted BWYV particles in Western blot and dot immunobinding assays. Varying amounts of purified BWYV samples (100-500 ng) were resuspended with an equal volume of SDS-PAGE sample buffer (4% SDS, 125 mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 0.4% bromophenol blue and 20% glycerol) and then incubated at 85°C for 10 min. Samples were centrifuged (13 000 x *g*) for 5 min and aliquots were electrophoresed through a 12% polyacrylamide gel using the buffer system of Laemmli (1970). Separated proteins were electroblotted (100V, 0.25A, 4°C, 1 hour) onto PVDF membrane (0.2 micron, BioRad) in a buffer composed of 25 mM Tris, 192 mM glycine, 20%

methanol (pH 8.3). The transfer blots were treated with blocking buffer (5% skim milk powder in 10 mM phosphate buffer, 150 mM NaCl) for 1 hour and incubated (4°C, overnight) first with the diluted monoclonal antibody of interest (in 10 mM Tris pH 7.5, 0.15 M NaCl, 1% BSA, 0.1% sodium azide, 0.05% Tween 20) or the polyclonal antisera and then [^{125}I]-labeled goat anti-mouse IgG or the [^{125}I]-labeled goat anti-rabbit IgG, respectively. The goat IgGs were radioiodinated by the chloramine T method (Sambrook *et al.*, 1989). After washing in PBS-Tween (10 mM phosphate buffer pH 7.5, 0.05% Tween 20) the membranes were exposed to X-ray film at -70°C overnight with intensifying screens. The specific activity of the radioiodinated IgG was approximately 5 x 10^7 cpm/mg protein.

Similarly, varying amounts of purified BWYV samples (50-600 ng) were spotted onto PVDF membrane. Untreated samples (intact BWYV particles) and treated samples (1% SDS, heating at 95°C for 5 min) were spotted onto the membranes and incubated with the monoclonal antibody or polyclonal antisera as above.

Results

3.1 Amplification of the BWYV Coat Protein Gene by Reverse Transcription - Polymerase Chain Reaction

Double-stranded (ds) complementary DNA (cDNA) copies of the BWYV coat protein gene were synthesized from BWYV RNA using reverse transcription (RT) followed by polymerase chain reaction (PCR). The BWYV RNA was extracted from purified virus, originally isolated from sugarbeet (provided by Peter Ellis, Agriculture Canada Vancouver Research Station). The first strand of the cDNA was synthesized utilizing reverse transcriptase and a 20-mer oligonucleotide (BWYV oligonucleotide #2, 5'-AGA AGG CCA TGG GCT AGG GC-3') primer. The sequence of the primer was based on the published genomic RNA sequence of BWYV (Veidt *et al.*, 1988). The BWYV oligonucleotide #2 is complementary to a region just downstream from the coat protein gene at nucleotides 4108-4127 of BWYV RNA. A nucleotide substitution in the primer sequence from a C to an A at position 4119 permitted the incorporation of a *Nco* I restriction enzyme site at the 3' end of the PCR product corresponding to the 3' terminus of the BWYV coat protein gene. The second strand of the cDNA, and the subsequent cDNA copies generated in the PCR reaction, were synthesized with a second 28-mer oligonucleotide (BWYV oligonucleotide #1, 5'-CGG CAC CAT GGA TAC GGT CGT GGG TAG G-3') primer. The BWYV

oligonucleotide #1 is identical to the sequence beginning at the coat protein initiator codon at nucleotides 3482-3503 of BWYV RNA except at position 3485. A nucleotide substitution in the primer sequence from an A to a G (highlighted in boldface) and the addition of seven nucleotides at the 5' end of the BWYV oligonucleotide #1 primer (underlined) permitted the incorporation of a *Nco* I restriction enzyme site at the 5' end of the expected PCR product. The resulting ds cDNA copy of the BWYV coat protein gene was 652 bp in size as determined by agarose gel electrophoresis (see Fig. 3.1, lane 5).

Initial attempts to synthesize double stranded DNA copies of the BWYV coat protein gene by RT-PCR from RNA extracts of BWYV-infected *Brassica napus*, collected from the U.B.C. field station (Ellis and Stace-Smith, 1990) were unsuccessful. PCR products were obtained from RNA extracts of both healthy and infected plants and were determined by agarose gel electrophoresis to be smaller in size than that expected for the cDNA of the BWYV coat protein gene (compare lanes 3 and 4 with lane 5, Fig. 3.1). Subsequent restriction enzyme analysis and partial nucleotide sequencing of the cloned products confirmed that they did not correspond to the BWYV coat protein gene nor to any other part of the BWYV genome (results not shown).

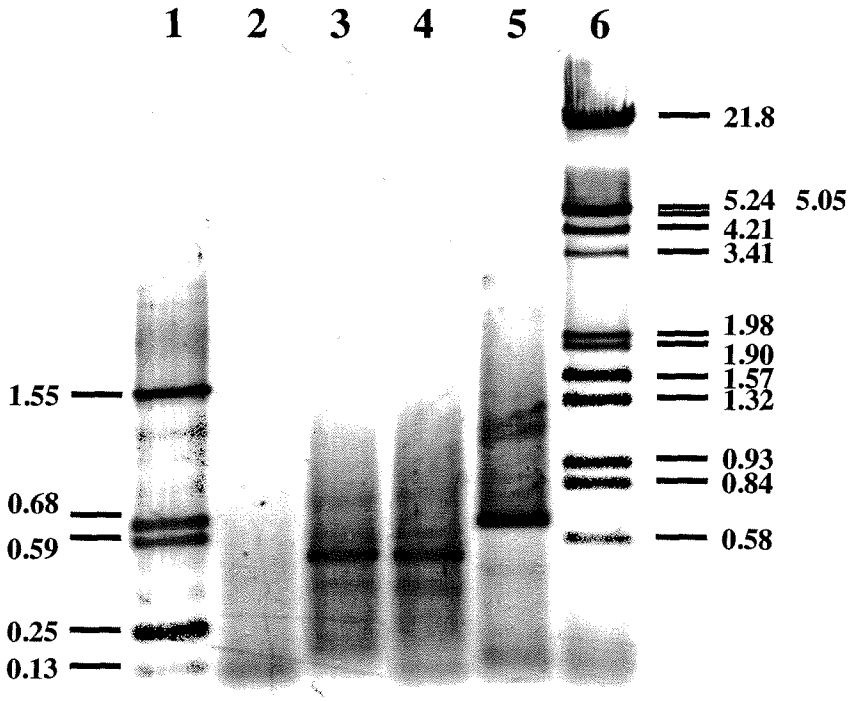


Fig. 3.1 Amplification of the BWYV coat protein gene by RT-PCR. The RNA templates used in the RT-PCR reactions were as follows: lane (2) contained no RNA template, lane (3) contained RNA from uninfected *Brassica napus*, lane (4) contained RNA from BWYV-infected *Brassica napus* and lane (5) contained BWYV RNA extracted from purified virus. The PCR products obtained were analyzed by electrophoresis through a 1% agarose gel and stained with ethidium bromide. Lanes (1) and (6) contained molecular size markers: pBluescribe digested with *Pvu* II and *Rsa* I; Lambda DNA digested with *Eco* RI and *Hin*d III (sizes in kbp).

3.2 Cloning of the Double-Stranded cDNA Copies of the BWYV Coat Protein Gene into the pRT103 Plant Expression Vector

The ds cDNA copy of the BWYV coat protein gene obtained by PCR amplification was digested with *Nco* I restriction enzyme and the compatible ends were ligated into the similarly digested pRT103 plasmid. The pRT103 plasmid carries the cauliflower mosaic virus (CaMV) 35*S* promoter and polyadenylation signal (Töpfer *et al.*, 1987; see Fig. 3.2). As well, the AUG codon of the *Nco* I restriction enzyme site (of the multicloning site) is embedded in the consensus sequence G/AxxAUGG for optimal ribosome initiation in eukaryotes (Kozak, 1984). The ligation mixture was used to transform competent *Escherichia coli* DH5α cells which were then grown on LB-agar containing ampicillin. Several ampicillin-resistant colonies were obtained. Individual colonies were cultured in Luria-Bertani (LB) broth containing ampicillin and their nucleic acid isolated by the small scale mini-preparation procedure. The presence and orientation of an insertion was determined by restriction enzyme mapping. The BWYV coat protein gene was determined by restriction enzyme mapping to be inserted into the *Nco*I site of the pRT103 plasmid in both the plus-sense orientation (clone BW102D) and the anti-sense orientation (clone BW137D) relative to the CaMV 35*S* promoter (see Fig. 3.2).

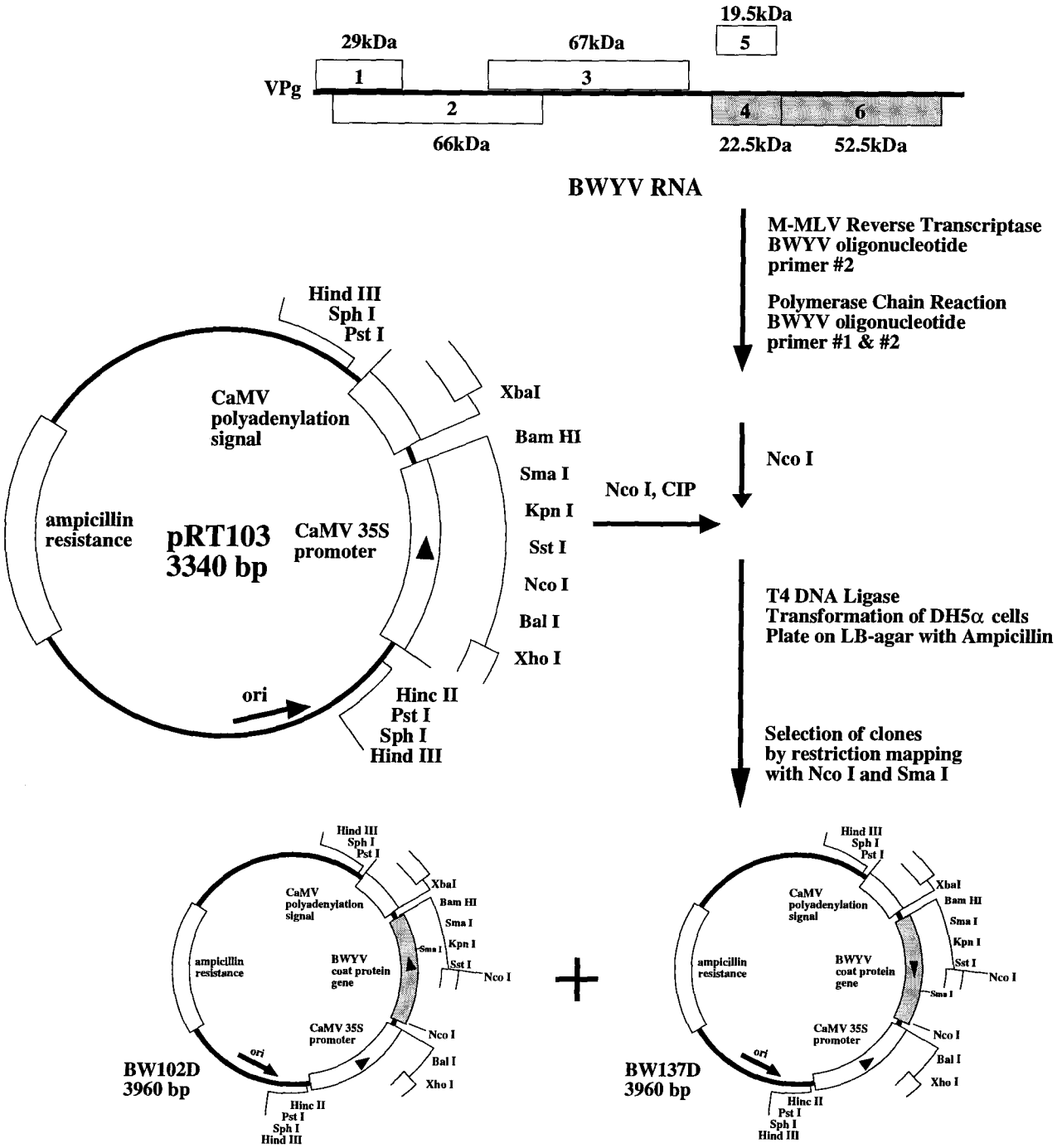


Fig. 3.2 Diagram showing the construction of the pRT103/BWYV coat protein gene clones. Purified viral RNA was used as template, together with the BWYV oligonucleotide primer #2, for the first strand cDNA synthesis using M-MLV reverse transcriptase. The second strand cDNA and subsequent copies, were synthesized by the addition of the BWYV oligonucleotide primer #1 in the polymerase chain reaction. Following amplification, the cDNA was digested with *Nco* I restriction enzyme and ligated into the similarly digested pRT103 plasmid using T4 DNA ligase. The ligation mixture was used to transform competent DH5αcells which were then plated on LB-agar containing ampicillin. The DNA from several individual colonies was screened by restriction enzyme mapping using *Nco* I and *Sma* I. The cDNA of the BWYV coat protein was found to be inserted into the *Nco* I site of the pRT103 plasmid in both the plus-sense (clone BW102D) and anti-sense orientation (clone BW137D) relative to the CaMV 35*S* promoter.

3.2.1 DNA Sequencing of the BWYV Coat Protein Gene

Both the pRT103 clones, containing the cDNA of the BWYV coat protein gene in the plus-sense orientation (clone BW102D) and in the anti-sense orientation (clone BW137D) relative to the CaMV 35*S* promoter, were sequenced using the same oligonucleotide primers (BWYV oligonucleotide #1, 5' primer and BWYV oligonucleotide #2, 3' primer) previously used in the PCR reactions (see section 3.1). In addition, two pRT103 primers were used to confirm the sequences flanking the *Nco* I site of insertion. The pRT103 oligonucleotide #1 primer is a 16-mer oligonucleotide (5'-CTT CCT CTA TAT AAG G-3') whose sequence is identical to a region of the CaMV 35S promoter upstream (5' end) from the multicloning site. The pRT103 oligonucleotide #2 primer is a 17-mer oligonucleotide (5'-CTA CTC ACA CAT TAT TC-3') whose sequence is complementary to a region downstream (3' end) from the multicloning site. Using these four primers (BWYV oligonucleotide #1 and #2, pRT103 oligonucleotide #1 and #2), the sequences at the 5'- and 3'-ends (approximately 250 bp on each end) of the BW102D and BW137D clones were determined (see Fig. 3.3, 3.4). The internal portion of the BWYV coat protein gene (approximately 200 bp) was sequenced using a *Sma* I deletion clone of BW102D. Clone BW102D, which contains the BWYV coat protein gene inserted in the plus-sense orientation, was digested with *Sma* I restriction enzyme. The larger portion of the plasmid (approximately 3.7 kbp in size) was gel purified and the ends re-ligated. The *Sma* I deletion clone (BW102DΔ*Sma* I) contained the BWYV coat protein gene

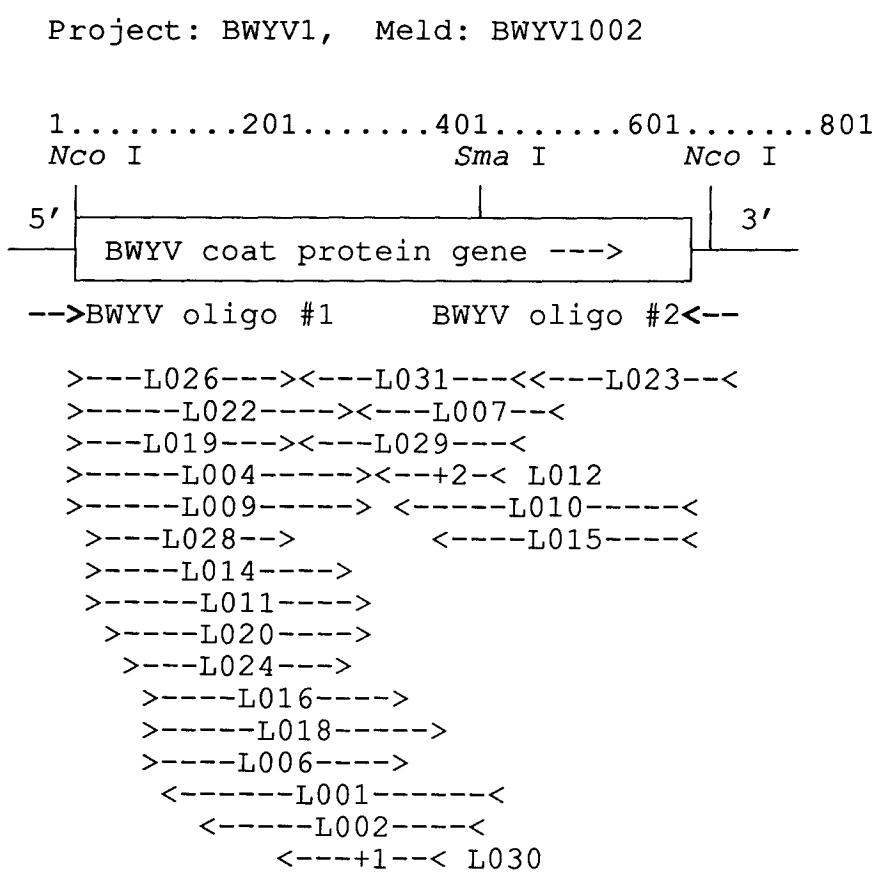


Fig. 3.3 Strategy used to sequence the BWYV coat protein coding region of clone BW102D. The 5' and 3' ends (250 bp) of the BWYV coat protein gene were sequenced using the BWYV oligonucleotide primers #1 and #2 and the pRT103 oligonucleotide primers #1 and #2. The internal portion of the BWYV coat protein gene (200 bp) was sequenced using a *Sma* I deletion clone of BW102D and the pRT103 oligonucleotide primer #2. A total of 23 different sequence readings from the two clones, BW102D and BW102DΔ*Sma* I, were used to obtain the consensus sequence BWYV1002 (650 bp). The arrows (>---> or <---<) indicate the direction of the sequence. (nn) represents the sequence number in L0nn.

```
         10         20         30         40
          |          |          |          |
CCACCATGGATACGGTCGTGGGTAGGAGAACAATCAATGGAAGAA

 P  P  W  I  R  S  W  V  G  E  Q  S  M  E  E
  H  H  G  Y  G  R  G  -  E  N  N  Q  W  K  K
   T  M  D  T  V  V  G  R  R  T  I  N  G  R

   50         60         70         80         90
    |          |          |          |          |
GACGACCACGCAGGCAAACACGACGCGCTCGGCCGTCTCAGCCAG

 D  D  H  A  G  K  H  D  A  L  G  R  L  S  Q
  T  T  T  Q  A  N  T  T  R  S  A  V  S  A  S
R  R  P  R  R  Q  T  R  R  A  R  P  S  Q  P

        100        110        120        130
          |          |          |          |
TGGTTGTGGTCCAAACCTCTCGGGCAACACAACGCCGACCTAGAC

 W  L  W  S  K  P  L  G  Q  H  N  A  D  L  D
  G  C  G  P  N  L  S  G  N  T  T  P  T  -  T
V  V  V  V  Q  T  S  R  A  T  Q  R  R  P  R

  140        150        160        170        180
    |          |          |          |          |
GACGACGAAGAGGTAATAACCGGACAAGAGGAACTGTTCCTACCA

 D  D  E  E  V  I  T  G  Q  E  E  L  F  L  P
  T  T  K  R  -  -  P  D  K  R  N  C  S  Y  Q
R  R  R  R  G  N  N  R  T  R  G  T  V  P  T

        190        200        210        220
          |          |          |          |
GAGGAGCAGGCTCAAGCGAGACATTTGTTTTCTCGAAAGACAATC

 E  E  Q  A  Q  A  R  H  L  F  S  R  K  T  I
  R  S  R  L  K  R  D  I  C  F  L  E  R  Q  S
R  G  A  G  S  S  E  T  F  V  F  S  K  D  N

  230        240        250        260        270
    |          |          |          |          |
TCGCGGGAAGTTCCAGCGGACGAATCACGTTCGGGCCGAGTCTAT

 S  R  E  V  P  A  D  E  S  R  S  G  R  V  Y
  R  G  K  F  Q  R  T  N  H  V  R  A  E  S  I
L  A  G  S  S  S  G  R  I  T  F  G  P  S  L
```

```
        280        290        300        310
          |          |          |          |
CAGACTGCCCAGCATTCTCTAATGGAATACTCAAGGCCTACCATG

 Q  T  A  Q  H  S  L  M  E  Y  S  R  P  T  M
  R  L  P  S  I  L  -  W  N  T  Q  G  L  P  -
S  D  C  P  A  F  S  N  G  I  L  K  A  Y  H

  320        330        340        350        360
    |          |          |          |          |
AGTATAAAATCTCGATGGTCATTTTGGAGTTCGTCTCCGAAGCCT

 S  I  K  S  R  W  S  F  W  S  S  S  P  K  P
  V  -  N  L  D  G  H  F  G  V  R  L  R  S  L
E  Y  K  I  S  M  V  I  L  E  F  V  S  E  A

        370        380        390        400
          |          |          |          |
CTTCCCAAAACTCCGGTTCCATCGCTTATGAGCTGGACCCACACT

 L  P  K  T  P  V  P  S  L  M  S  W  T  H  T
  F  P  K  L  R  F  H  R  L  -  A  G  P  T  L
S  S  Q  N  S  G  S  I  A  Y  E  L  D  P  H

  410        420        430        440        450
    |          |          |          |          |
GTAGACTCGACGCCCTTTCCTCGACCATCAATAAGTTCGGGATCA

 V  D  S  T  P  F  P  R  P  S  I  S  S  G  S
  -  T  R  R  P  F  L  D  H  Q  -  V  R  D  H
C  R  L  D  A  L  S  S  T  I  N  K  F  G  I

        460        470        480        490
          |          |          |          |
CAAAGCCCGGGAGGAGGGCGTTTACAGCGTCTTACATCAACGGGA

 Q  S  P  G  G  G  R  L  Q  R  L  T  S  T  G
  K  A  R  E  E  G  V  Y  S  V  L  H  Q  R  D
T  K  P  G  R  R  A  F  T  A  S  Y  I  N  G

  500        510        520        530        540
    |          |          |          |          |
CGGATTGGCACGACGTTGCCAAGGACCAATTCAGGATCCTCTACA

 R  I  G  T  T  L  P  R  T  N  S  G  S  S  T
  G  L  A  R  R  C  Q  G  P  I  Q  D  P  L  Q
T  D  W  H  D  V  A  K  D  Q  F  R  I  L  Y
```

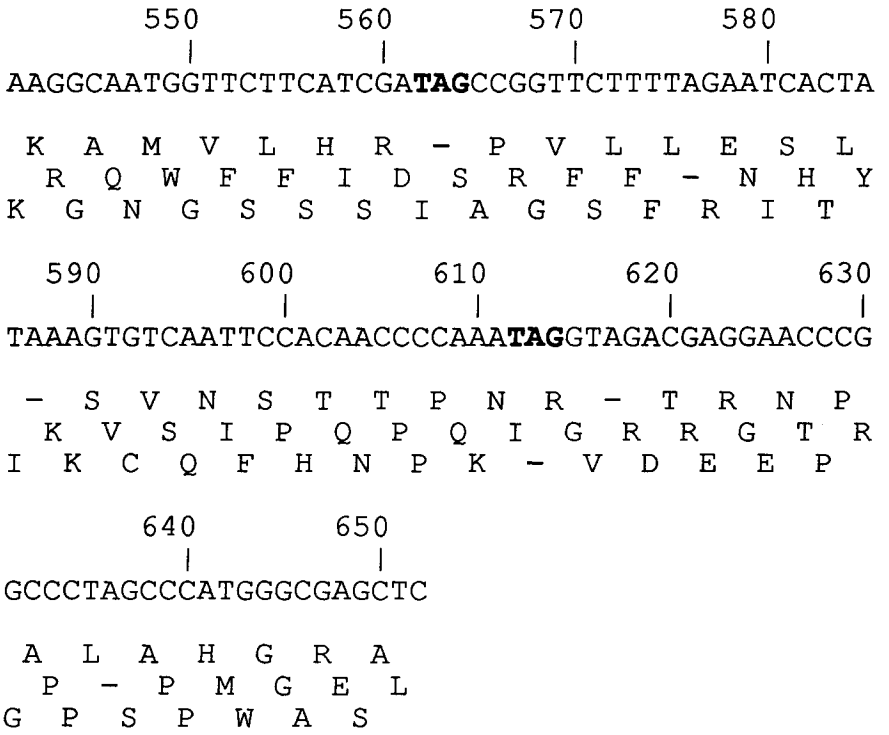


Fig. 3.4 Nucleotide sequence and amino acid sequence of the BWYV coat protein coding region. The sequence of the BWYV coat protein cDNA was constructed from a meld of 23 different sequence readings of clone BW102D in the ASSEMGEL program of PCGENE (IntelliGenetics). The first and second initiation codons, **ATG**,correspond to the BWYV 22.5 kDa coat protein and the 19.5 kDa putative genome-linked protein (VPg), respectively, and are highlighted in boldface. Also shown is the predicted amino acid sequence in the three different open reading frames (ORF). The dashes in the amino acid sequence correspond to stop codons in the nucleotide sequence. The terminator codon, **TAG**, for each ORF is also shown in boldface. Single letter codes were used for the various amino acids.

with approximately 200 bp deleted from the 3' end of the coat protein gene. Since the *Nco* I site at the 3' end of the coat protein gene was also deleted, it was necessary to use the pRT103 oligonucleotide #2 primer to sequence the internal portion of the coat protein (see Fig. 3.3). The sequences obtained from the clones corresponded closely to the published coat protein gene sequence from a BWYV isolate collected from sugarbeet (Veidt *et al.*, 1988) and confirmed that no reading frame errors had been introduced during the PCR reaction. The nucleotide sequence of the BWYV coat protein cDNA (BWYV1002) shared 94.2% and 93.5% sequence identity with the sugarbeet isolate, BWYV (GB1) and a lettuce isolate, BWYV (FL1), respectively (see Figs. 3.5, 3.6). A comparison of the protein sequences revealed 94.1% and 92.6% identity with the same sugarbeet isolate and lettuce isolate, respectively (see Figs. 3.7, 3.8). Furthermore, the AUG codon of the BWYV coat protein gene was confirmed to be embedded in the consensus sequence for optimal ribosome initiation, G/AxxAUGG where x represents any nucleotide (Kozak, 1984).

```
BWYV(GB1)  - CGTTAATGAATACGGTCGTGGGTAGGAGAACAATCAATGG -40
             |  *  |||*|||||||||||||||||||||||||||||||||
BWYV1002   - CCACCATGGATACGGTCGTGGGTAGGAGAACAATCAATGG -40

BWYV(GB1)  - AAGAAGACGACCACGCAGGCAAACACGACGCGCTCAGCGC -80
             |||||||||||||||||||||||||||||||||||||| ||
BWYV1002   - AAGAAGACGACCACGCAGGCAAACACGACGCGCTCGGCCG -80

BWYV(GB1)  - TCTCAGCCAGTGGTTGTGGTCCAAACCTCTCGGGCAACAC -120
             ||||||||||||||||||||||||||||||||||||||||
BWYV1002   - TCTCAGCCAGTGGTTGTGGTCCAAACCTCTCGGGCAACAC -120

BWYV(GB1)  - AACGCCGACCTAGACGACGACGAAGAGGTAACAACCGGAC -160
             |||||||||||||||||||||||||||||| ||||||||
BWYV1002   - AACGCCGACCTAGACGACGACGAAGAGGTAATAACCGGAC -160

BWYV(GB1)  - AAGAGGAACTGTTCCTACCAGAGGAGCAGGCTCGAGCGAG -200
             |||||||||||||||||||||||||||||| ||||||
BWYV1002   - AAGAGGAACTGTTCCTACCAGAGGAGCAGGCTCAAGCGAG -200

BWYV(GB1)  - ACATTTGTTTTCTCAAAAGACAATCTCGCGGGAAGTTCCA -240
             |||||||||||||| ||||||||||||||||||||||||
BWYV1002   - ACATTTGTTTTCTCGAAAGACAATCTCGCGGGAAGTTCCA -240

BWYV(GB1)  - GCGGACGAATCACGTTCGGGCCGAGTCTATCAGACTGCCC -280
             |||||||||||||||||||||||||||||||||||||||
BWYV1002   - GCGGACGAATCACGTTCGGGCCGAGTCTATCAGACTGCCC -280

BWYV(GB1)  - GGCATTCTCTAATGGAATGCTCAAGGCCTACCATGAGTAT -320
              |||||||||||||||| ||||||||||||||||||||
BWYV1002   - AGCATTCTCTAATGGAATACTCAAGGCCTACCATGAGTAT -320

BWYV(GB1)  - AAAATCTCAATGGTCATTTTGGAGTTCGTCTCCGAAGCCT -360
             |||||||| ||||||||||||||||||||||||||||||
BWYV1002   - AAAATCTCGATGGTCATTTTGGAGTTCGTCTCCGAAGCCT -360

BWYV(GB1)  - CTTCCCAAAACTCCGGTTCCATCGCTTACGAGCTGGACCC -400
             |||||||||||||||||||||||||| ||||||||||||
BWYV1002   - CTTCCCAAAACTCCGGTTCCATCGCTTATGAGCTGGACCC -400

BWYV(GB1)  - ACACTGTAAACTCAACTCCCTTTCCTCAACTATCAACAAG -440
             |||||||| |||| || |||||||||| || ||||| |||
BWYV1002   - ACACTGTAGACTCGACGCCCTTTCCTCGACCATCAATAAG -440

BWYV(GB1)  - TTCGGGATCACAAAGCCCGGGAAAGCGGCGTTTACAGCGT -480
             |||||||||||||||||||||    ||||||||||||||
BWYV1002   - TTCGGGATCACAAAGCCCGGGAGGAGGGCGTTTACAGCGT -480

BWYV(GB1)  - CTTACATCAATGGAAAGGAATGGCACGACGTTGCCGAGGA -520
             |||||||||| || | ||| |||||||||||||| ||||
BWYV1002   - CTTACATCAACGGGACGGATTGGCACGACGTTGCCAAGGA -520
```

```
BWYV(GB1)  - CCAATTCAGGATCCTCTACAAAGGCAATGGTTCTTCATCG -560
             |||||||||||||||||||||||||||||||||||||||
BWYV1002   - CCAATTCAGGATCCTCTACAAAGGCAATGGTTCTTCATCG -560

BWYV(GB1)  - ATAGCTGGTTCTTTTAGAATCACCATCAAGTGCCAATTCC -600
             |||||||||||||||||||||||||||||||||||||||||
BWYV1002   - ATAGCCGGTTCTTTTAGAATCACTATAAAGTGTCAATTCC -600

BWYV(GB1)  - ACAATCCCAAATAGGTAGACGAGGAACCCGGCCCTAGCCC -640
             |||||||||||||||||||||||||||||||||||||||||
BWYV1002   - ACAACCCCAAATAGGTAGACGAGGAACCCGGCCCTAGCCC -640

BWYV(GB1)  - A-GGGCCTTCT  -650
             |*||||   ||
BWYV1002   - ATGGGCGAGCTC -652
```

Fig. 3.5 Nucleotide sequence comparison between the BWYV coat protein coding region (BWYV1002 meld) and the coat protein sequence of the sugarbeet isolate (Veidt *et al.*, 1988), BWYV (GB1). The first and second **ATG** codons correspond to the initiation codon for the BWYV 22.5 kDa coat protein and 19.5 kDa ORFs, respectively. The character to show that two aligned residues are identical is 'l'. Nucleotide changes introduced via the BWYV oligonucleotide primers are indicated by a '*'. The terminator codon, **TAG**, for each ORF is shown in boldface.

```
BWYV(FL1)  - CGTTAATGAATACGGTCGTGGGTAGGAGAATTATCAATGG -40
             |  *  |||*|||||||||||||||||||||  ||||||||
BWYV1002   - CCACCATGGATACGGTCGTGGGTAGGAGAACAATCAATGG -40

BWYV(FL1)  - AAGAAGACGACCACGCAGGCAAACACGACGCGCTCAGCGC -80
             |||||||||||||||||||||||||||||||||||||| ||
BWYV1002   - AAGAAGACGACCACGCAGGCAAACACGACGCGCTCGGCCG -80

BWYV(FL1)  - CCTCAGCCAGTGGTTGTGGTCCAAACCTCTCGGGCAACAC -120
              ||||||||||||||||||||||||||||||||||||||||
BWYV1002   - TCTCAGCCAGTGGTTGTGGTCCAAACCTCTCGGGCAACAC -120

BWYV(FL1)  - AACGCCGACCTAGACGACGACGAAGAGGTAACAACCGGAC -160
             |||||||||||||||||||||||||||||| ||||||||
BWYV1002   - AACGCCGACCTAGACGACGACGAAGAGGTAATAACCGGAC -160

BWYV(FL1)  - AGGAAGAACTGTTCCTACCAGAGGAGCAGGTTCGAGCGAG -200
             | ||  ||||||||||||||||||||||||| ||  ||||||
BWYV1002   - AAGAGGAACTGTTCCTACCAGAGGAGCAGGCTCAAGCGAG -200

BWYV(FL1)  - ACATTTGTTTTCTCAAAAGACAATCTCGCGGGAAGTTCCA -240
             |||||||||||||| ||||||||||||||||||||||||
BWYV1002   - ACATTTGTTTTCTCGAAAGACAATCTCGCGGGAAGTTCCA -240

BWYV(FL1)  - GCGGAGCAATCACGTTCGGGCCGAGTCTATCAGACTGCCC -280
             |||||  |||||||||||||||||||||||||||||||||
BWYV1002   - GCGGACGAATCACGTTCGGGCCGAGTCTATCAGACTGCCC -280

BWYV(FL1)  - GGCATTCTCTAATGGAATGCTCAAGGCCTACCATGAGTAT -320
              ||||||||||||||||| ||||||||||||||||||||
BWYV1002   - AGCATTCTCTAATGGAATACTCAAGGCCTACCATGAGTAT -320

BWYV(FL1)  - AAAATCTCAATGGTCATTTTGGAGTTCGTCTCCGAAGCCT -360
             |||||||| |||||||||||||||||||||||||||||||
BWYV1002   - AAAATCTCGATGGTCATTTTGGAGTTCGTCTCCGAAGCCT -360

BWYV(FL1)  - CTTCCCAAAATTCCGGTTCCATCGCTTACGAGCTGGACCC -400
             |||||||||| |||||||||||||||| ||||||||||
BWYV1002   - CTTCCCAAAACTCCGGTTCCATCGCTTATGAGCTGGACCC -400

BWYV(FL1)  - ACACTGTAAACTCAACTCCCTTTCCTCAACTATCAACAAG -440
             |||||||| |||| || |||||||||| || ||||| |||
BWYV1002   - ACACTGTAGACTCGACGCCCTTTCCTCGACCATCAATAAG -440

BWYV(FL1)  - TTCGGGATCACAAAGCCCGGGAAAAGGGCGTTTACAGCGT -480
             |||||||||||||||||||||  ||||||||||||||||
BWYV1002   - TTCGGGATCACAAAGCCCGGGAGGAGGGCGTTTACAGCGT -480

BWYV(FL1)  - CTTACATCAACGGAACGGAATGGCACGACGTTGCCGAGGA -520
             |||||||||||| ||||| |||||||||||||| ||||
BWYV1002   - CTTACATCAACGGGACGGATTGGCACGACGTTGCCAAGGA -520
```

```
BWYV(FL1)  - CCAATTCAGGATCCTCTACAAAGGCAATGGTTCTTCATCG -560
             |||||||||||||||||||||||||||||||||||||||
BWYV1002   - CCAATTCAGGATCCTCTACAAAGGCAATGGTTCTTCATCG -560

BWYV(FL1)  - ATAGCTGGTTCTTTCAGAATCACCATTAAGTGTCAATTCC -600
             |||||  ||||||||  ||||||||  ||  ||||||||||||
BWYV1002   - ATAGCCGGTTCTTTTAGAATCACTATAAAGTGTCAATTCC -600

BWYV(FL1)  - ACAACCCCAAATAGGTAGACGAGGAACCCGGCCCTAGCCC -640
             |||||||||||||||||||||||||||||||||||||||||
BWYV1002   - ACAACCCCAAATAGGTAGACGAGGAACCCGGCCCTAGCCC -640

BWYV(FL1)  - A-GGGCCTTCT  -650
             |*||||   ||
BWYV1002   - ATGGGCGAGCTC -652
```

Fig. 3.6 Nucleotide sequence comparison between the BWYV coat protein coding region (BWYV1002 meld) and the coat protein sequence of the lettuce isolate (Veidt *et al.*, 1988), BWYV (FL1). The first and second **ATG** codons correspond to the initiation codons for the BWYV 22.5 kDa coat protein and 19.5 kDa ORF, respectively. The character to show that two aligned residues are identical is '|'. Nucleotide changes introduced via the BWYV primers are indicated by a '*'. The terminator codon, **TAG**, for each ORF is shown in boldface.

```
BWYV(GB1)  - MNTVVGRRTINGRRRPRRQTRRAQRSQPVVVVQTSRATQR -40
             | ||||||||||||||||||||||  ||||||||||||||||
BWYV1002   - MDTVVGRRTINGRRRPRRQTRRARPSQPVVVVQTSRATQR -40

BWYV(GB1)  - RPRRRRRGNNRTRGTVPTRGAGSSETFVFSKDNLAGSSSG -80
             ||||||||||||||||||||||||||||||||||||||||
BWYV1002   - RPRRRRRGNNRTRGTVPTRGAGSSETFVFSKDNLAGSSSG -80

BWYV(GB1)  - RITFGPSLSDCPAFSNGMLKAYHEYKISMVILEFVSEASS -120
             |||||||||||||||| ||||||||||||||||||||||
BWYV1002   - RITFGPSLSDCPAFSNGILKAYHEYKISMVILEFVSEASS -120

BWYV(GB1)  - QNSGSIAYELDPHCKLNSLSSTINKFGITKPGKAAFTASY -160
             |||||||||||||| |  ||||||||||||||  ||||||
BWYV1002   - QNSGSIAYELDPHCRLDALSSTINKFGITKPGRRAFTASY -160

BWYV(GB1)  - INGKEWHDVAEDQFRILYKGNGSSSIAGSFRITIKCQFHN -200
             |||  ||||| ||||||||||||||||||||||||||||||
BWYV1002   - INGTDWHDVAKDQFRILYKGNGSSSIAGSFRITIKCQFHN -200

BWYV(GB1)  - PK -202
             ||
BWYV1002   - PK -202
```

Fig. 3.7 Amino acid sequence comparison between the BWYV coat protein (derived from BWYV1002 meld) and the coat protein of the sugarbeet isolate (Veidt *et al.*, 1988), BWYV (GB1). The numbering begins from the methionine which corresponds to the first **ATG** in Fig . The character to show that two aligned residues are identical is 'l'

```
BWYV(FL1)  - MNTVVGRRIINGRRRPRRQTRRAQRPQPVVVVQTSRATQR -40
             | |||||| ||||||||||||||    ||||||||||||||
BWYV1002   - MDTVVGRRTINGRRRPRRQTRRARPSQPVVVVQTSRATQR -40

BWYV(FL1)  - RPRRRRRGNNRTGRTVPTRGAGSSETFVFSKDNLAGSSSG -80
             ||||||||||||  ||||||||||||||||||||||||||
BWYV1002   - RPRRRRRGNNRTRGTVPTRGAGSSETFVFSKDNLAGSSSG -80

BWYV(FL1)  - AITFGPSLSDCPAFSNGMLKAYHEYKISMVILEFVSEASS -120
              ||||||||||||||||| ||||||||||||||||||||||
BWYV1002   - RITFGPSLSDCPAFSNGILKAYHEYKISMVILEFVSEASS -120

BWYV(FL1)  - QNSGSIAYELDPHCKLNSLSSTINKFGITKPGKRAFTASY -160
             |||||||||||||| |  |||||||||||||| |||||||
BWYV1002   - QNSGSIAYELDPHCRLDALSSTINKFGITKPGRRAFTASY -160

BWYV(FL1)  - INGTEWHDVAEDQFRILYKGNGSSSIAGSFRITIKCQFHN -200
             |||| ||||| ||||||||||||||||||||||||||||||
BWYV1002   - INGTDWHDVAKDQFRILYKGNGSSSIAGSFRITIKCQFHN -200

BWYV(FL1)  - PK -202
             ||
BWYV1002   - PK -202
```

Fig. 3.8 Amino acid sequence comparison between the BWYV coat protein (derived from BWYV1002 meld) and the coat protein of the lettuce isolate (Veidt *et al.*, 1988), BWYV (FL1). The numbering begins from the methionine which corresponds to the first ATG in Fig. 3.6. The character to show that two aligned residues are identical is 'l'

3.3 *In Vitro* Translation Analysis of Synthetic Transcripts of the Cloned BWYV Coat Protein Gene

3.3.1 Cloning of the BWYV Coat Protein Gene into a Transcription Vector and the *In Vitro* Transcription of the BWYV Coat Protein Gene using the Bacteriophage T3 Polymerase

The BWYV coat protein gene was obtained by digestion of BW102D with *Xho* I/*Sst* I restriction enzymes. The 654 bp fragment corresponding to the BWYV coat protein gene was excised from pRT103 and gel purified. The compatible ends were ligated with similarly digested pBluescriptII KS+ plasmid (Stratagene) such that the 5' portion of the BWYV coat protein gene was adjacent to the bacteriophage T3 RNA polymerase promoter. The ligation mixture was used to transform competent *E. coli* DH5α cells which were then plated on LB-agar containing ampicillin and Bluo-Gal (BRL). Several white ampicillin-resistant colonies were obtained. Individual colonies were cultured in LB broth containing ampicillin and their nucleic acid isolated by the small scale mini-preparation procedure. The presence and orientation of an insertion was determined by restriction enzyme mapping. The pBluescript/BWYV coat protein gene construct was linearized at the 3' end of the BWYV coat protein gene with *Sst* I restriction enzyme and used to make synthetic RNA transcripts *in vitro* with the T3 RNA polymerase.

3.3.2 *In Vitro* Translation of Synthetic BWYV Coat Protein RNA in Wheat Germ Extracts

Synthetic RNA transcripts were translated in wheat germ extracts in the presence of [^{35}S]-methionine and the protein products were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent autoradiography (see Fig. 3.9). The size of the protein product corresponded to the expected size of the BWYV coat protein (22.5 kDa) and not to the product encoded by ORF 5 (19.5 kDa).

3.4 Construction of the BWYV Coat Protein Gene Binary Plasmids Using the pCGN15 Vector Series (Calgene)

Clones BW102D and BW137D were digested with *Hind* III restriction enzyme (see Fig. 3.10 and 3.11). The appropriate fragments of approximately 1.3 kbp in size were gel purified and the compatible ends re-ligated into the *Hind* III digested pCGN1548 and pCGN1557 binary vectors (McBride and Summerfelt, 1990). The 1.3 kbp fragments excised from either clone BW102D or clone BW137D contained the CaMV 35*S* promoter, the BWYV coat protein gene (in the plus-sense orientation or the anti-sense orientation, respectively) and the CaMV polyadenylation signal. The pCGN1548 and pCGN1557 binary vectors contain the *npt* II (neomycin phosphotransferase) gene as a plant selectable marker regulated by the mannopine synthase (*mas* 5') promoter / termination signal (*mas* 3') and by the CaMV 35*S* promoter / *tml*

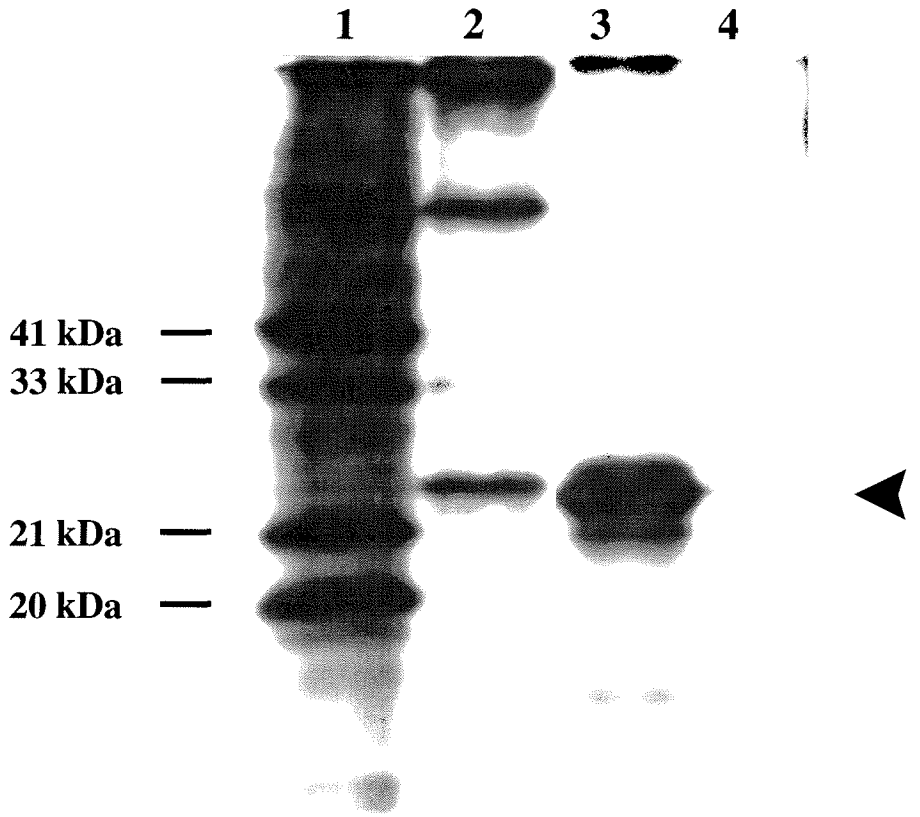


Fig. 3.9 *In vitro* translation analysis of the synthetic transcripts of the cloned BWYV coat protein gene. The BWYV coat protein cDNA was cloned next to the T3 promoter of pBluescriptII KS+ plasmid. The Bluescript/BWYV coat protein gene construct was linearized with *Sst* I restriction enzyme and used to make synthetic RNA transcripts in an *in vitro* transcription run off reaction. Synthetic RNA transcripts were translated in wheat germ extracts in the presence of [^{35}S]-methionine and the protein products were analyzed by denaturing polyacrylamide gel electrophoresis (15% gel) and subsequent autoradiography. The following templates were used in the *in vitro* translation reactions: lanes (1) cucumber necrosis virus (CNV) RNA (50 ng) used as a protein molecular weight standard , (2) BWYV RNA extracted from purified virus (100 ng), (3) BWYV coat protein synthetic transcript (50 ng) and (4) no RNA. The arrow points to the BWYV coat protein. The high molecular weight band in lane 2 likely corresponds to the BWYV 66 kDa, encoded by ORF 2. The numbers on the left correspond to the sizes of CNV *in vitro* translation products.

Fig. 3.10 Diagram showing the construction of the pCGN1548/BWYV coat protein gene clones. BW102D and BW137D clones were digested with *Hind* III restriction enzyme in separate reactions. The 1.3 kbp *Hind* III fragment was gel purified from both of the clones and ligated into the *Hind* III site of the binary plasmid pCGN1548 (Calgene) using T4 DNA ligase. The ligation mixture was used to transform competent DH5α cells which were then plated on LB-agar containing ampicillin and Bluo-gal (BRL). The DNA from several individual white colonies was screened by restriction enzyme mapping using *Sma* I and *Xho* I. The *Hind* III fragment containing the CaMV 35*S* promoter/polyadenylation signal and the BWYV coat protein gene in the plus-sense orientation and the anti-sense orientation were successfully inserted into the Hind III site of pCGN1548; making the pCGN154802 and pCGN154837 binary plasmid constructs, respectively. The restriction enzyme sites for *Eco* RI (E), *Hind* III (H), *Sma* I (S) and *Xho* I (X) are shown. See section 1.5 for a description of the components of the pCGN15 binary plasmids. (CIP= calf intestinal phosphatase; *npt* II= Tn5 neomycin phosphotransferase gene; Gm= gentamycin resistance marker; ori pRI= origin of replication of the *A. rhizogenes* root inducing plasmid pRiHRI; RB/LB= right /left border regions from pTiA6; *mas* 5'= mannopine synthase promoter from pTiA6; *mas* 3'= mannopine synthase termination signals from pTiA6; *tml* 3'= *tml* terminator from pTiA6; ori Col E1= origin of replication and transfer origin from pBR322; lac Z'= *E. coli* lac alpha polylinker from pUC18)

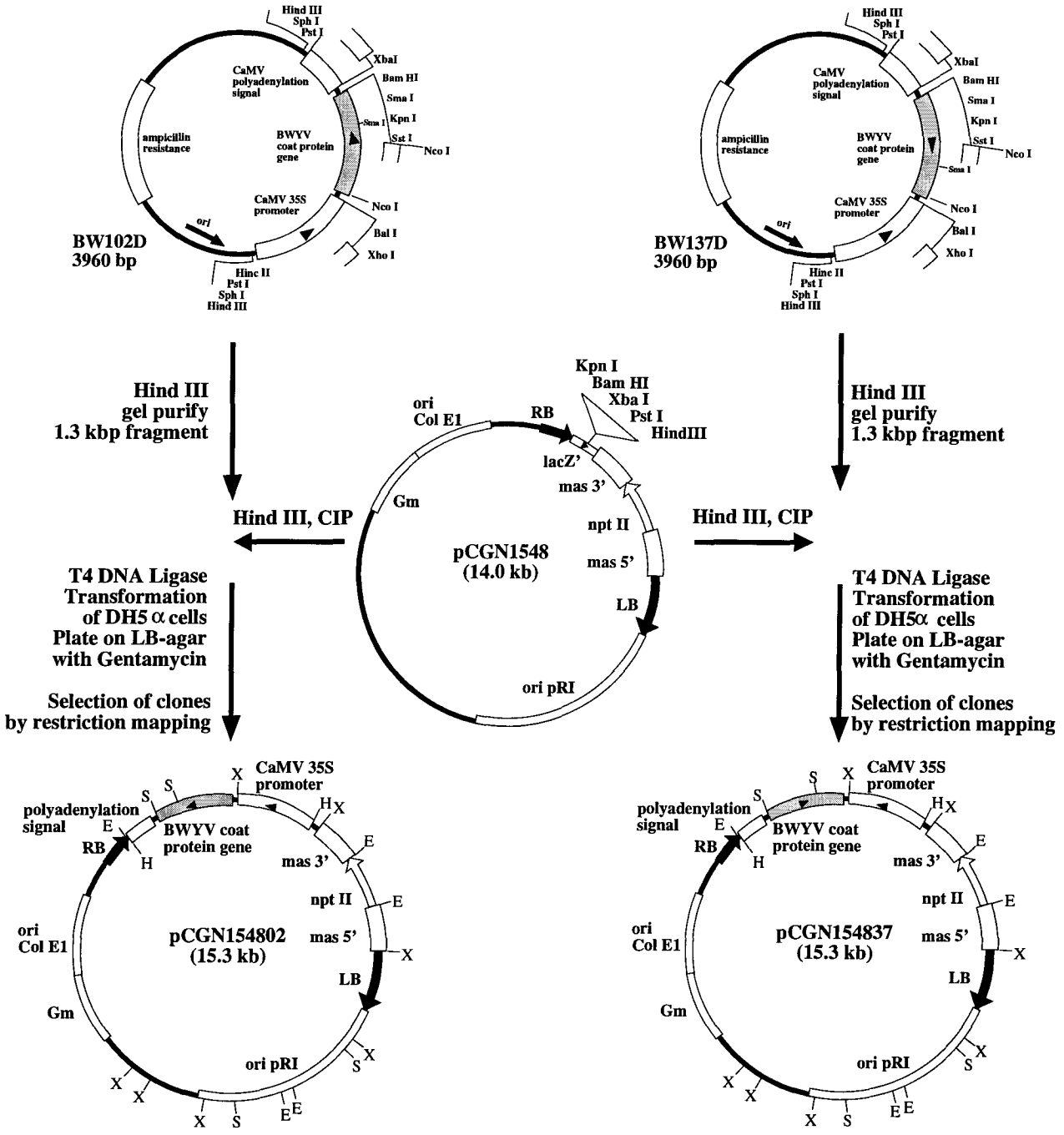
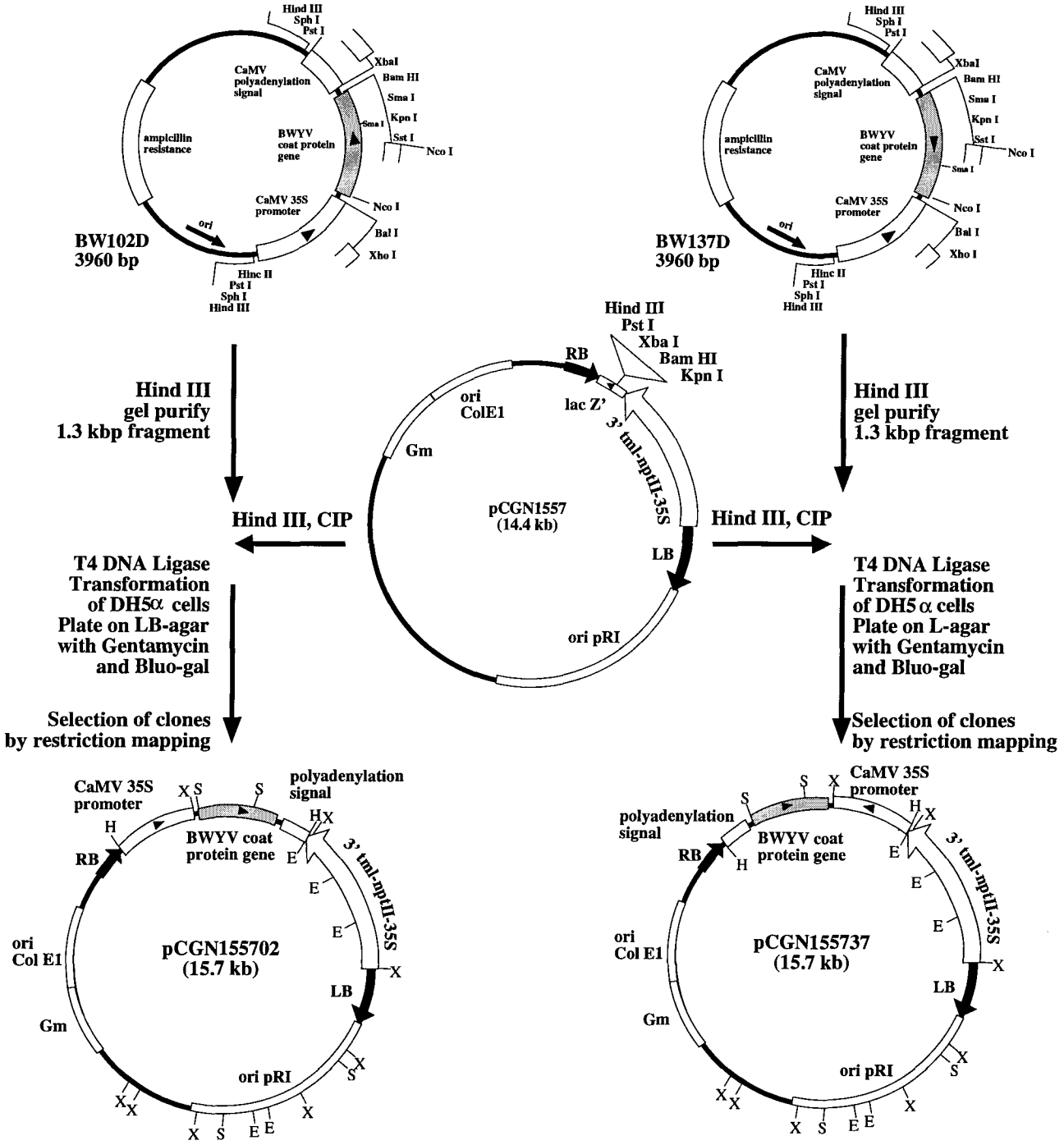

BW102D
3960 bp
Hind III
Sph I
Pst I
XbaI
Bam HI
Sma I
Kpn I
Sst I
Nco I
CaMV polyadenylation signal
ampicillin resistance
BWYV coat protein gene
CaMV 35S promoter
ori
Nco I
Bal I
Xho I
Hinc II
Pst I
Sph I
Hind III
BW137D
3960 bp
Hind III gel purify 1.3 kbp fragment
Hind III, CIP
T4 DNA Ligase Transformation of DH5 α cells Plate on LB-agar with Gentamycin
Selection of clones by restriction mapping
Kpn I
Bam HI
Xba I
Pst I
HindIII
ori Col E1
RB
lacZ'
mas 3'
Gm
npt II
pCGN1548
(14.0 kb)
mas 5'
LB
ori pRI
pCGN154802
(15.3 kb)
pCGN154837
(15.3 kb)
polyadenylation signal
BWYV coat protein gene
CaMV 35S promoter

Fig. 3.11 Diagram showing the construction of the pCGN1557/BWYV coat protein gene clones. BW102D and BW137D clones were digested with *Hind* III restriction enzyme in separate reactions. The 1.3 kbp *Hind* III fragment was gel purified from both of the clones and ligated into the *Hind* III site of the binary plasmid pCGN1557 (Calgene) using T4 DNA ligase. The ligation mixture was used to transform competent DH5α cells which were then plated on LB-agar containing ampicillin and Bluo-gal (BRL). The DNA from several individual white colonies was screened by restriction enzyme mapping using Sma I and *Xho* I. The *Hind* III fragment containing the CaMV 35*S* promoter/polyadenylation signal and the BWYV coat protein gene in the plus-sense orientation and the anti-sense orientation were successfully inserted into the *Hind* III site of pCGN1557; making the pCGN155702 and pCGN155737 binary plasmid constructs, respectively. The restriction enzyme sites for *Eco* RI (E), *Hind* III (H), *Sma* I (S) and *Xho* I (X) are shown. (CIP= calf intestinal phosphatase; *npt* II= Tn5 neomycin phosphotransferase gene; Gm= gentamycin resistance marker; ori pRI= origin of replication of the *A. rhizogenes* root inducing plasmid pRiHRI; RB/LB= right /left border regions from pTiA6; *mas* 5'= mannopine synthase promoter from pTiA6; *mas* 3'= mannopine synthase termination signals from pTiA6; *tml* 3'= *tml* terminator from pTiA6; ori Col E1= origin of replication and transfer origin from pBR322; lac Z'= *E. coli* lac alpha polylinker from pUC18)


Hind III
Sph I
Pst I
XbaI
Bam HI
Sma I
Kpn I
Sst I
Nco I
CaMV polyadenylation signal
ampicillin resistance
BWYV coat protein gene
CaMV 35S promoter
ori
Bal I
Xho I
Hinc II
BW102D
3960 bp
BW137D
3960 bp
Hind III gel purify 1.3 kbp fragment
Hind III, CIP
T4 DNA Ligase Transformation of DH5α cells Plate on LB-agar with Gentamycin and Bluo-gal
Selection of clones by restriction mapping
Hind III
Pst I
Xba I
Bam HI
Kpn I
RB
ori ColE1
lac Z'
3' tml-nptII-35S
Gm
pCGN1557
(14.4 kb)
LB
ori pRI
Hind III, CIP
T4 DNA Ligase Transformation of DH5 α cells Plate on L-agar with Gentamycin and Bluo-gal
Selection of clones by restriction mapping
CaMV 35S promoter
polyadenylation signal
BWYV coat protein gene
RB
ori Col E1
pCGN155702
(15.7 kb)
Gm
LB
ori pRI
3' tml-nptII-35S
pCGN155737
(15.7 kb)

terminator (*tml* 3'), respectively. The ligation mixture was used to transform competent *E. coli* DH5α cells which were then plated on LB-agar containing the antibiotic gentamycin and the chromogenic substrate Bluo-Gal (BRL). Several white gentamycin-resistant colonies were obtained. Individual colonies were cultured in Luria-Bertani (LB) broth containing gentamycin and their nucleic acid isolated by the small scale mini-preparation procedure. The presence and orientation of an insertion was determined by restriction enzyme mapping. Several clones were obtained (see Fig. 3.12): pCGN154802 and pCGN155702 containing the BWYV coat protein gene in the plus-sense orientation relative to the CaMV 35*S* promoter; pCGN154837 and pCGN155737 containing the BWYV coat protein gene in the anti-sense orientation relative to the CaMV 35S promoter.

3.5 Mobilization of the BWYV Coat Protein Gene Binary Plasmids into *Agrobacterium tumefaciens* EHA101 by the Tri-Parental Mating Procedure

The binary plasmid constructs pCGN154802, pCGN154837, pCGN155702 and pCGN155737 were used to transform *E. coli* MM294 cells which were then plated on LB-agar containing gentamycin. The binary plasmids pCGN1548 and pCGN1557 were also used to transform *E. coli* MM294 cells as controls. Several gentamycin-resistant colonies were obtained. Individual colonies of each construct were cultured in LB broth containing gentamycin. Cultures containing the appropriate construct were co-cultured with *E. coli* MM294 containing the pRK2013 helper plasmid and

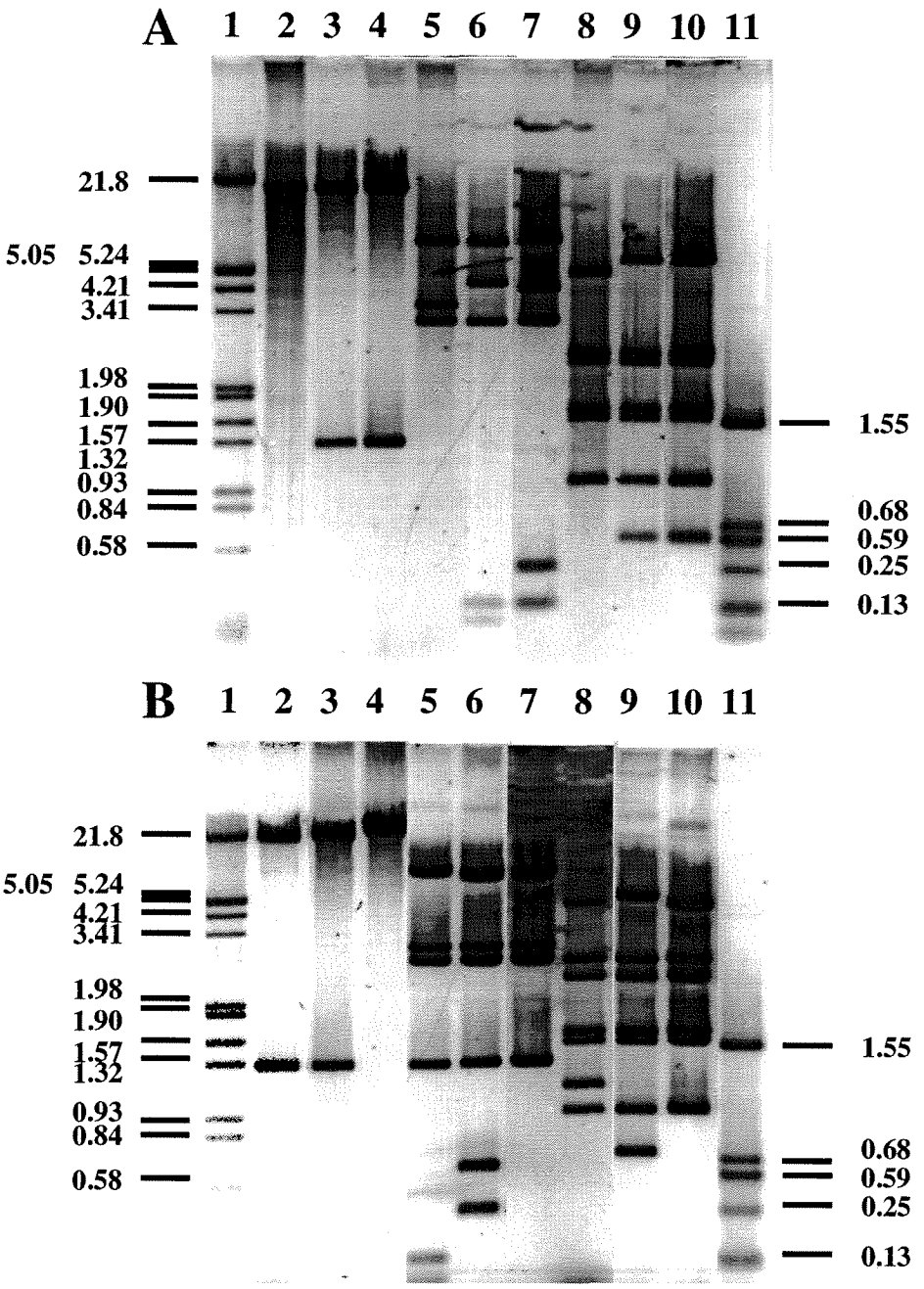


Fig. 3.12 Restriction enzyme analysis of pCGN1548- and pCGN1557-derived BWYV coat protein gene constructs. Plasmid constructs, containing the BWYV coat protein gene in the plus-sense orientation (relative to the CaMV 35*S* promoter) - pCGN154802 (A,lanes 3,6,9) and pCGN155702 (B, lanes 2,5,8) - and in the anti-sense orientation (relative to the CaMV 35*S* promoter) - pCGN154837 (A, lanes 4, 7, 10) and pCGN155737 (B, lanes 3, 6, 9) - were digested with *Hin* d III (lanes 2-4),*Sma* I (lanes 5-7) and *Xho* I (lanes 8-10) restriction enzymes. Plasmids not containing the BWYV coat protein gene were similarly digested as controls - pCGN1548 (A, lanes 2, 5, 8) and pCGN1557 (B, lanes 4, 7, 10). The resulting products were analyzed by electrophoresis through a 1% agarose gel and stained with ethidium bromide. Lanes 1 and 11 contain molecular size markers: Lambda DNA digested with *Eco* RI and *Hin*d III and pBluescribe digested with *Pvu* II and *Rsa* I (sizes shown in kbp), respectively.

Agrobacterium tumefaciens EHA101 (Hood *et al.*, 1986) and grown on LB-agar containing both antibiotics, gentamycin and kanamycin. Several gentamycin and kanamycin-resistant *A. tumefaciens* colonies were obtained. The presence of the appropriate binary plasmid construct could not be determined by small scale nucleic acid preparation of the *A. tumefaciens* and subsequent restriction enzyme mapping since the yield of plasmid was too low. But the presence of the appropriate binary plasmid was confirmed by digestion of the nucleic acid preparation with *Hin*d III restriction enzyme, size fractionated by agarose gel electrophoresis and subsequent Southern blot analysis using a random-primed, [^{32}P]-labeled cDNA BWYV coat protein gene probe (results not shown). Nucleic acid preparations from the *A. tumefaciens* containing the appropriate binary plasmid construct were also used to transform competent *E. coli* DH5α cells. The cells were then plated onto LB-agar containing gentamycin and Bluo-gal (BRL). Several white gentamycin-resistant colonies were obtained, cultured and their nucleic acid isolated by the small scale mini-preparation procedure. The presence of the appropriate binary plasmid construct was confirmed by restriction enzyme mapping.

3.6 Transformation and Regeneration of *Brassica napus* cv. Westar

Brassica napus cv. Westar was transformed with the appropriate binary plasmid constructs according to the plant transformation procedure utilizing cotyledonary explants (Moloney *et al.*, 1989). The *A. tumefaciens* EHA101 used in the

transformation procedure contained one of the following binary plasmid constructs: pCGN154802, pCGN154837, pCGN155702 and pCGN155737. As a control, some cotyledons were not treated with *A. tumefaciens* in order to determine the base level of regeneration in untreated *B. napus* cv. Westar under the plant tranformation conditions. In all, 5280 cotyledonary explants were used in four separate attempts to transform *B. napus* cv. Westar (see Table 3.1). Several regenerated, kanamycin-resistant shoots were subcultured and eventually placed in the greenhouse where they were allowed to flower and set seed. Shoots that had not been transformed with the *npt* II gene appeared bleached by the fourth week of culture. The regeneration efficiency of the cotyledonary explants after treatment with the *A. tumefaciens* ranged from 0.8 to 3.6% as compared to 17 to 80% in the untreated cotyledonary explants (see Table 3.3). Thirty-eight regenerated plants were recovered from the first two transformation attempts: five which had been treated with the *A. tumefaciens* containing the pCGN154802 binary plasmid construct, one with the pCGN154837 binary plasmid construct, three with the pCGN1548 binary plasmid, fifteen with the pCGN155702 binary plasmid construct, six with the pCGN155737 binary plasmid construct and eight treated with the *A. tumefaciens* containing the pCGN1557 binary plasmid. All the regenerated plants appeared morphologically identical to untreated *B. napus* cv. Westar except for plant 155702-9, which was dwarfish and sterile. Several modifications implemented in the third (July 1992) and fourth (September 1992) attempts could not be evaluated since *A. tumefaciens* used in the plant transformation procedure reappeared and contaminated all the regenerated shoots. The reappearance

Table 3.1 Transformation of *Brassica napus* cv. Westar with *Agrobacterium tumefaciens* EHA101 containing the indicated binary plasmid construct. Four separate attempts were made following the plant transformation procedure using cotyledonary explants (Moloney *et al.*, 1989). (---) indicates that no cotyledons were treated on this date.

	Number of *Brassica napus* cv. Westar cotyledons treated in the plant transformation procedure					
Agrobacterium /constructs	Jan.24/92	Apr.5/92	Apr.6/92	Jul.9/92	Sept.23/92	Totals
EHA101/ pCGN155702	170	110	140	360	400	1180
EHA101/ pCGN155737	170	110	130	360	400	1170
EHA101/ pCGN1557	170	110	---	50	---	330
EHA101/ pCGN154802	---	---	140	360	400	900
EHA101/ pCGN154837	---	---	140	360	400	900
EHA101/ pCGN1548	---	---	140	50	---	190
no *Agrobacterium*	190	110	130	120	60	610
Totals	700	440	820	1540	1660	5280

Table 3.2 Transformation of *Brassica juncea* cv. Forge with *Agrobacterium tumefaciens* EHA101 containing the indicated binary plasmid construct. An attempt was made to transform *Brassica juncea* cv. Forge, using the plant transformation procedure (Moloney *et al.*, 1989), for a comparison of the regeneration efficiency for a different species. (---) indicates that no cotyledons were treated on this date.

Agrobacterium / constructs	Number of *Brassica juncea* cv. Forge cotyledons treated in transformation procedure				
	Jan.24/92		Apr.6/92		Totals
	number of explants treated	regeneration efficiency %	number of explants treated	regeneration efficiency %	
EHA101 / pCGN155702	20	0.0	100	0.0	120
EHA101 / pCGN155737	20	0.0	---	---	20
EHA101 / pCGN1557	20	0.0	---	---	20
no *Agrobacterium*	28	0.0	90	0.0	118
Totals	88	0.0	190	0.0	278

Table 3.3 Regeneration efficiency of *Brassica napus* cv. Westar cotyledons after co-cultivation of the cotyledonary explants with *Agrobacterium tumefaciens* EHA101 containing the appropriate binary plasmid construct. (---) indicates that no cotyledons were treated on this date.

Agrobacterium /constructs	Date regenerated *Brassica napus* cv. Westar placed in greenhouse					
	April to June		July		October	
	number regenerated	regeneration efficiency %	number regenerated	regeneration efficiency %	number regenerated	regeneration efficency %
EHA 101 / pCGN155702	6 / 170*	3.5	9 / 250	3.6	2 / 360	0.6
EHA 101 / pCGN155737	4 / 170	2.4	2 / 240	0.8	0 / 360	0.0
EHA 101 / pCGN1557	4 / 170	2.4	4 / 110	3.6	0 / 50	0.0
EHA 101 / pCGN154802	---	---	5 / 140	3.6	0 / 360	0.0
EHA101 / pCGN154837	---	---	1 / 140	0.7	0 / 360	0.0
EHA 101 / pCGN1548	---	---	3 / 140	2.1	1 / 50	2.0
Totals	14 / 510	2.7	24 / 1020	2.4	3 / 1560	0.2
no *Agrobacterium*	152 / 190	80	156 / 240	65	20 / 120	17

* number of plants regenerated / number of cotyledons treated.

was likely due to a reduction in the concentration of antibiotics used during the shoot rooting stage or to an excessive initial inoculum of *A. tumefaciens*. Modifications (suggested by Sharon E. Radke, Calgene and Gijs H. van Rooijen, University of Calgary) to the plant transformation procedure included: (1) co-cultivation of the *A. tumefaciens* (reduced inoculum) with excised cotyledons for 24 hours instead of 72 hours, (2) use of kanamycin throughout the transformation procedure in order to reduce the number of possible "escapes" (kanamycin sensitive shoots) and (3) use of cefotaxime instead of or in combination with carbenicillin to inhibit the reappearance of the *A. tumefaciens* after the regenerated shoots have been subcultured. *Brassica juncea* cv. Forge was also used in the plant transformation procedure but no regenerated shoots were recovered. This confirms that the plant transformation procedure used (Moloney *et al.*, 1989) is genotype specific for *Brassica napus* (see Table 3.2).

3.7 Evaluation of Regenerated Plants

3.7.1 Southern Blot Analysis of DNA Extracted from Regenerated Plants

Plant DNA was isolated from regenerated plants by the CTAB extraction method. Approximately 50 to 150 ug of plant DNA sample (the amount depended on the final concentration of DNA obtained in the extraction) was digested with either *Hin*d III or *Eco* RI restriction enzyme and size fractionated by agarose gel

electrophoresis. The DNA was transferred onto Zeta-Probe GT membrane (BioRad) by alkaline capillary blotting. The Southern blots were probed with either random primed, [^{32}P]-labeled DNA corresponding to the *npt* II gene or to the BWYV coat protein gene.

Probing of the Southern blots, of *Hin*d III digested DNA, with [^{32}P]-labeled BWYV coat protein cDNA revealed a 1.3 kbp fragment in the plant DNA samples from three plants (154802-1, 154802-3 and 1557-7; see Fig. 3.13, lanes 5, 7 and 4, respectively). Hybridization to a 1.3 kbp fragment containing the CaMV 35*S* promoter/BWYV coat protein gene (plus-sense and anti-sense orientation)/CaMV polyadenylation signal occurred as predicted to *Hin*d III digested DNA from the appropriate binary plasmid constructs (pCGN154802, pCGN154837, pCGN155702 and pCGN155737; Fig. 3.13, lanes 16, 17, 13 and 14, respectively) but did not occur as expected to *Hin*d III digested binary plasmids used as controls (pCGN1548 and pCGN1557; Fig. 3.13, lanes 18 and 15, respectively). Hybridization of the BWYV coat protein cDNA probe to *Hin*d III digested DNA from plant 1557-7 (supposedly a binary plasmid not containing the BWYV coat protein gene) suggested that the plant had been, in fact, transformed with a BWYV coat protein gene binary plasmid construct. Plant 1557-7 was likely labeled incorrectly. Probing of *Eco* RI digested plant DNA with random primed, [^{32}P]-labeled DNA corresponding to the *npt* II gene revealed a 1.0 kbp fragment in the DNA sample from one plant (1557-7) and a 2.5 kbp fragment in the DNA sample from another plant (155737-5; see Fig. 3.14 A, lanes

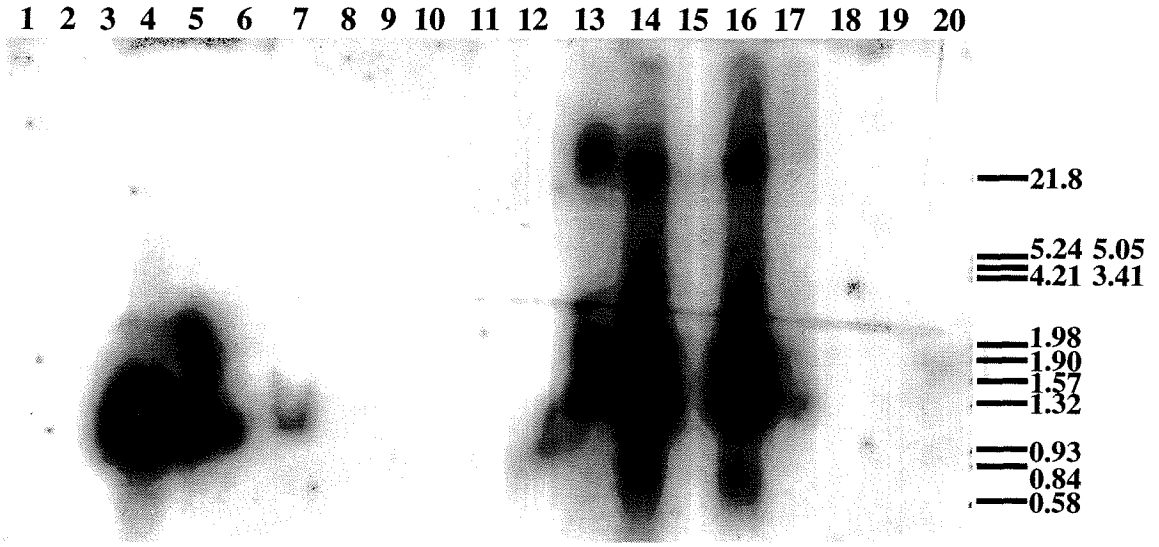


Fig. 3.13 Southern blot analysis of DNA extracted from regenerated *Brassica napus* cv. Westar. Approximately 50-150 ug of genomic DNA extracted from regenerated plants was digested with *Hin*d III restriction enzyme and electrophoresed through a 1% agarose gel. The size fractionated *Hin* d III digested genomic DNA was transferred onto Zeta-Probe GT membrane (BioRad) by alkaline capillary blotting. The membranes were probed with random primed, [^{32}P]-labeled BWYV coat protein cDNA and autoradiographed. Lanes 1-2 contained *Hin*d III digested genomic DNA corresponding to plants 155737-5 to -6 (ie. plants transformed with construct 155737), lanes 3-4 to plants 1557-5 and -7, lanes 5-9 to plants 154802-1 to -5, lane 10 to plant 154837-1 and lanes 11-12 to plants 1548-1 and -2. In addition, lane 20 contained *Hin*d III digested genomic DNA extracted from an untransformed plant. The controls included: *Hin*d III digested pCGN155702, pCGN155737, pCGN1557, pCGN154802, pCGN154837, and pCGN1548 plasmid DNA (approx. 25 pg, lanes 13-18, respectively). Lane 19 contained Lambda DNA digested with *Eco* RI and *Hin* d III used as a molecular size marker (sizes shown in kbp on right).

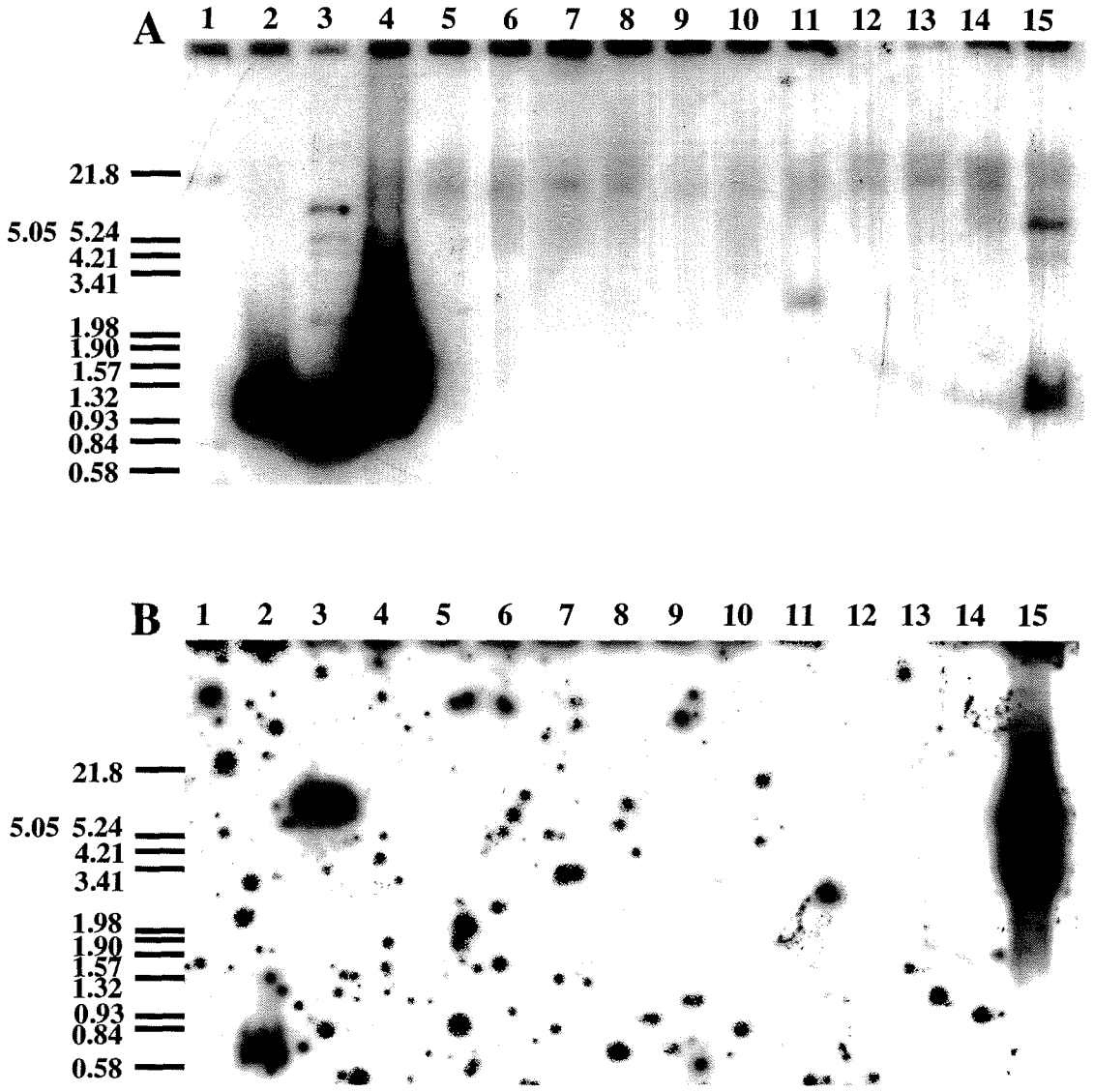


Fig. 3.14 Southern blot analysis of DNA extracted from regenerated *Brassica napus* cv. Westar. Approximately 50-150 ug of genomic DNA extracted from regenerated plants was digested with *Eco*RI restriction enzyme and electrophoresed through a 1% agarose gel. The size fractionated digested genomic DNA was transferred onto Zeta-Probe GT membrane (BioRad) by alkaline capillary blotting. The membranes were probed with random primed, [^{32} P]-labeled *npt* II cDNA (blot A) or BWYV coat protein cDNA (blot B) and autoradiographed. Lanes 6-9 contained *Eco* RI digested genomic DNA corresponding to plants 155702-12 to -15, lanes 10-12 to plants 155737-4 to -6 and lanes 13-15 to plants 1557-5 to -7. The controls included: *Eco*RI digested plasmid DNA pCGN155737 and pCGN1557 (lanes 3,4), *Eco*RI digested genomic DNA extracted from an untransformed plant (lane 5) and 25 pg each of *npt* II cDNA and BWYV coat protein cDNA mixed in *Eco*RI digested genomic DNA (lane 2). Lane 1 contained Lambda DNA digested with *Eco*RI and *Hin*d III used as molecular size marker (sizes shown in kbp).

15 and 11, respectively). A 1.0 kbp fragment containing the *npt* II gene occurred as predicted from *Eco* RI digestion of all binary plasmid constructs including the binary plasmids used as controls. The 2.5 kbp fragment (DNA sample from plant 155737-5; see Fig. 3.14A, lane 11) is likely to be the result of incomplete digestion of the *Eco* RI site between the *npt* II gene and the *tml* terminator (*tml* 3' region). Probing of the same Southern blots, after stripping of the *npt* II cDNA probe from the blot, with random primed [^{32}P]-labeled DNA corresponding to the BWYV coat protein gene produced a fragment (> 5.0 kbp) in the DNA sample from plant 1557-7 and a similar fragment (but weaker signal) in the DNA sample from plant 154802-3 (see Fig. 3.14B, lane 15; Fig. 3.15B, lane 8, respectively). A fragment no smaller than 2.3 kbp was predicted from *Eco* RI digestion of the binary plasmid constructs (pCGN154802, pCGN154837, pCGN155702and pCGN155737). The BWYV coat protein gene in pCGN155702 and pCGN155737 is flanked by only one *Eco* RI restriction site and therefore, the size of the fragment revealed in the Southern blot analysis would depend on the left border integrating next to a plant *Eco* RI restriction site. Genomic DNA from plant 154802-3 was also digested with *Hin*d III and hybridization of the probe corresponding to the BWYV coat protein gene revealed a 1.3 kbp fragment (see Fig. 3.15B, lane 9), as predicted from digestion of all the binary plasmid constructs. Since hybridization was found to occur with *Hin*d III digested DNA from plant 154802-1 using the probe corresponding to the BWYV coat protein gene (see Fig. 3.13, lane 5), it was surprising to find that no hybridization occurred with *Eco* RI digested DNA from plant 154802-1 (see Fig. 3.15B, lane 6) using the BWYV coat protein probe .

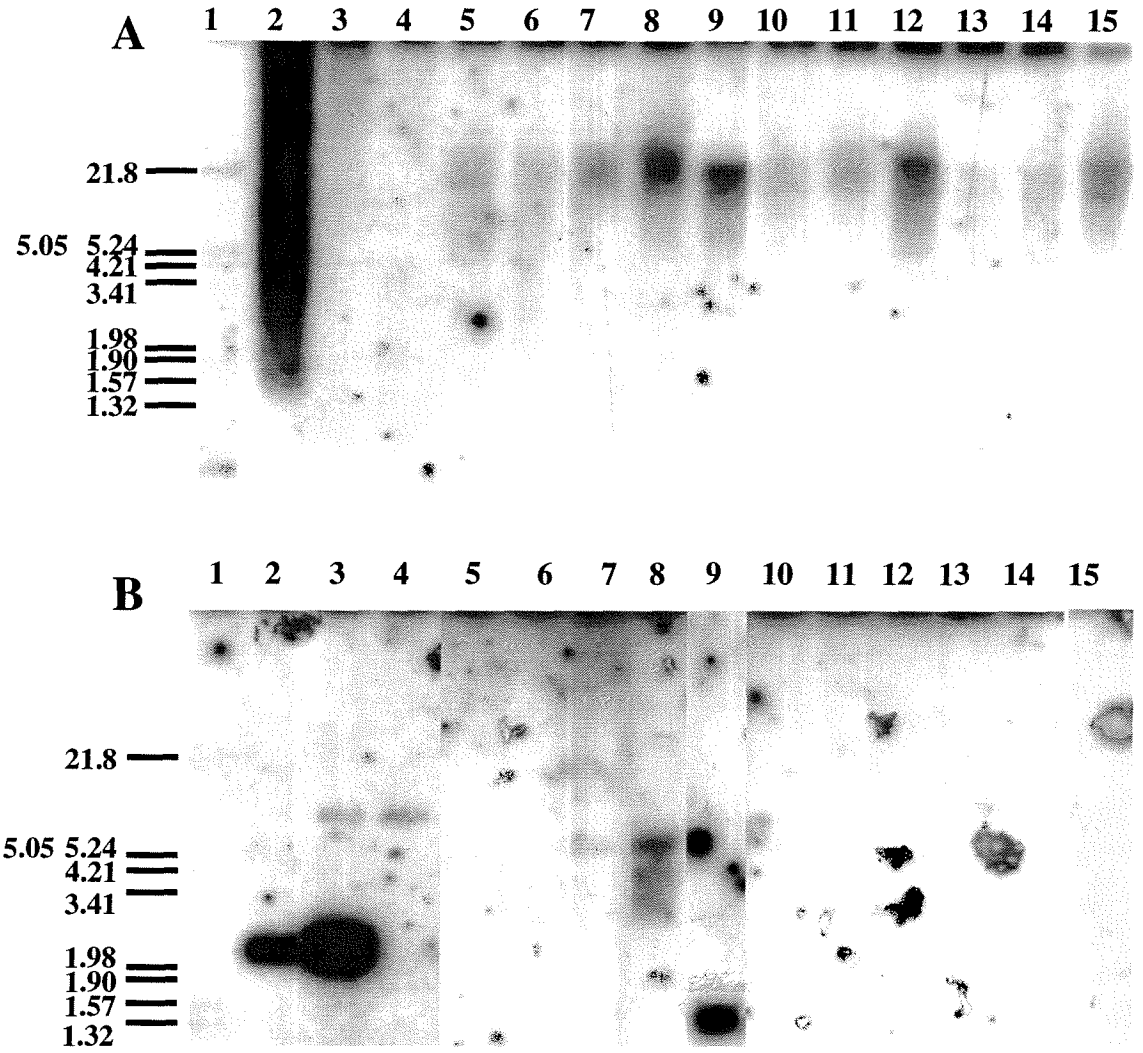


Fig. 3.15 Southern blot analysis of DNA extracted from regenerated *Brassica napus* cv. Westar. Approximately 50-150 ug of genomic DNA extracted from regenerated plants was digested with *Eco*RI restriction enzyme and electrophoresed through a 1% agarose gel. The size fractionated *Eco*RI digested genomic DNA was transferred onto Zeta-Probe GT membrane (BioRad) by alkaline capillary blotting. The membranes were probed with random primed, [^{32}P]- labeled *npt* II cDNA (blot A) or BWYV coat protein cDNA (blot B) and autoradiographed. Lane 5 contained *Eco*RI digested genomic DNA corresponding to plant 1557-8, lanes 6-8,10,11 to plants 154802-1 to -5, lane 12 to plant 154837-1 and lane 13-15 to plants 1548-1 to -3. In addition, lane 9 contained *Hin*d III digested genomic DNA corresponding to plant 154802-3. The controls included: *Eco* RI digested plasmid DNA of pCGN154802, pCGN154837, and pCGN1548 (lanes 2-4, respectively). Lane 1 contained Lambda DNA digested with *Eco*RI and *Hin*d III used as molecular size marker (sizes shown in kbp).

Similarly, hybridization was found to occur with *Eco* RI digested DNA from plant 155737-5 using the probe corresponding to the *npt* II gene (see Fig. 3.14A, lane 11) but no hybridization occurred with *Eco* RI digested DNA from plant 155737-5 (see Fig. 3.14B, lane 11) using the BWYV coat protein probe. No hybridization of BWYV coat protein cDNA probe was expected nor occurred for the binary plasmids (pCGN1548 and pCGN1557) used as controls.

The results from Southern blot analysis of *Hin*d III digested plant genomic DNA corresponding to the regenerated plants suggests, although not conclusively, that three plants have been successfully transformed with the BWYV coat protein gene (plants 154802-1, 154802-3 and 1557-7). Plant 1557-7, which supposedly had been transformed with the binary plasmid 1557 containing no BWYV coat protein gene, was likely incorrectly labeled. Furthermore, Southern blot analysis of *Eco* RI digested genomic DNA indicated that two plants had been successfully co-transformed with the *npt* II gene (plants 155737-5 and 1557-7). Surprisingly, the *npt* II gene could not be detected in Southern blot analysis of the genomic DNA from plants 154802-1 and 154802-3, for which the presence of the BWYV coat protein gene had been indicated in *Hin*d III digests (*Eco* RI digested plant DNA produced a signal only for the plant 154802-3). Similarly, the BWYV coat protein gene could not be detected in Southern blot analysis of the genomic DNA from plant 155737-5, for which the presence of the *npt* II gene had been indicated and presumed to be present since the plant was kanamycin resistant (see Discussion).

3.7.2 Northern Blot Analysis of RNA Extracted from Regenerated Plants

Total leaf RNA was extracted from all regenerated plants except for plant 155702-9, which was stunted and sterile. Approximately 10 to 40 ug of RNA was size fractionated by electrophoresis through glyoxal containing or MeHgOH-containing agarose gels and transferred onto Zeta-Probe GT membrane by alkaline capillary blotting. The Northern blots were probed with either random primed, [^{32}P]-labeled DNA corresponding to the *npt* II gene or to the BWYV coat protein gene. In three separate attempts, RNA transcripts of the *npt* II gene and BWYV coat protein gene could not be detected in the RNA samples extracted from any of the regenerated plants (results not shown). Both controls were detected in Northern blot analysis; they included 100 pg of BWYV RNA extracted from purified virus and 50 pg of synthetic BWYV coat protein transcript.

3.7.3 PCR Analysis of DNA Extracted from Regenerated Plants

The polymerase chain reaction (PCR) was used for detection of the BWYV coat protein gene in DNA samples extracted from regenerated plants. A PCR primer based on the CaMV 35*S* promoter sequence, 5'-CAC TAT CCT TCG CAA GAC CCT TCC TC-3' (35S-1 primer kindly supplied by Robert R. Martin, Agriculture Canada Vancouver Research Station) was used in combination with either of the original PCR primers (BWYV oligonucleotide #1 or #2) used to synthesize the BWYV coat protein

gene cDNA. In the DNA samples from plants expected to contain the BWYV coat protein gene in the plus-sense orientation relative to the CaMV 35*S* promoter, the 35S-1 primer was used with BWYV oligonucleotide primer #2 in the PCR reaction. In the DNA samples from plants expected to contain the BWYV coat protein gene in the anti-sense orientation, the 35S-1 primer was used with BWYV oligonucleotide primer #1 in the PCR reaction. Finally, in the DNA samples from untransformed plants or plants that contained the control binary plasmid, the 35S-1 primer was used with both BWYV oligonucleotide primers #1 and #2. The expected size of the PCR product using the 35S-1 primer in combination with either the BWYV oligonucleotide primer #1 or #2 is approximately 720 bp. The PCR products were analyzed by agarose gel electrophoresis followed by Southern blot analysis using a random primed, [^{32}P]-labeled DNA probe corresponding to the BWYV coat protein gene.

The Southern blot analysis was used to confirm that the PCR products corresponded to the BWYV coat protein gene (as opposed to an unrelated product found previously with RT-PCR from both healthy and BWYV-infected plants, see Section 3.1). Southern blot analysis of the PCR products revealed a 720 bp fragment in several plant DNA samples (see Fig. 3.16). When the PCR products were electrophoresed through a 1% agarose gel and visualized by staining with ethidium bromide it was found that in some lanes the most prominent band was about 500 bp in

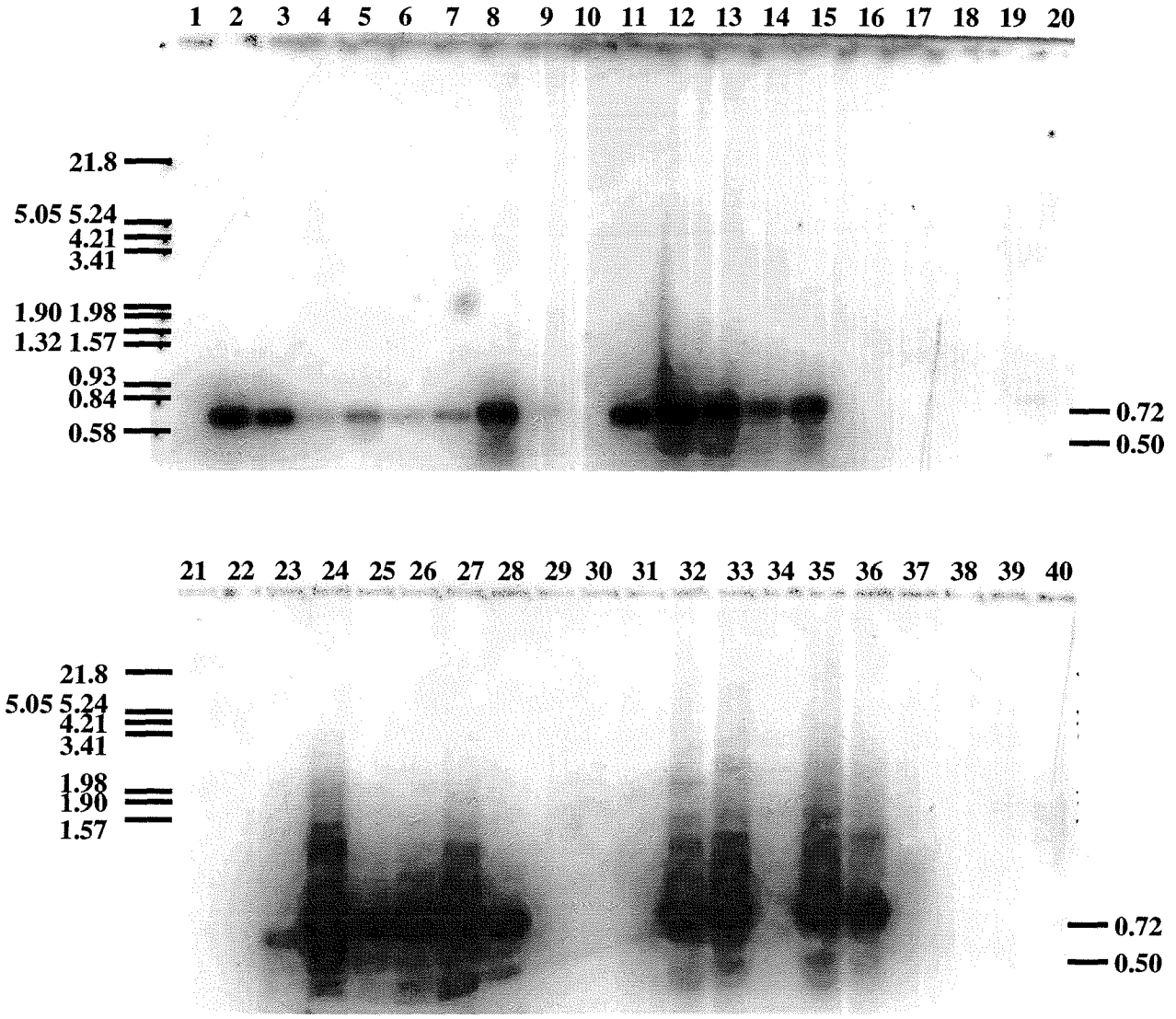


Fig. 3.16 Southern blot analysis of the PCR products obtained using DNA extracted from regenerated plants as template. Template DNA used in the PCR reactions was extracted from the following regenerated plants: 155702-1 to -15 (lanes 2-16), 155737-1 to -6 (lanes 17-22), 1557-7 (lane 23), 154802-1 to -5 (lanes 24-28), 154837-1 (lane 29) and 1548-2 to -3 (lanes 30,31). The PCR products obtained were analyzed by electrophoresis through a 1% agarose gel and stained with ethidium bromide. The PCR products were transferred onto Zeta-Probe GT membrane (BioRad) by alkaline capillary blotting. The membranes were probed with random primed, [^{32}P]-labeled BWYV coat protein cDNA and autoradiographed. Templates used in the PCR reactions as controls included the binary plasmids pCGN155702, pCGN155737, pCGN1557, pCGN154802, pCGN154837 and pCGN1548 (lanes 32-37, respectively). Plant DNA extracted from uninfected *Brassica napus* cv. Westar (lane 39) and water (lane 40) were also included as controls. Lanes 1 and 38 contain molecular size markers (in kbp): Lambda DNA digested with *Eco* RI and *Hin*d III. The numbers on the right indicate the expected size of the coat protein PCR product, 720 bp and of the 500 bp non-specific band, likely a result of mis-priming using the CaMV 35S-1 primer.

size (see Fig. 3.17, lanes 3-7). This band likely resulted from mis-priming using the 35S-1 primer. Previously, an attempt to amplify the BWYV coat protein gene and the *npt* II gene as a single fragment in constructs containing two CaMV 35*S* promoters (see Fig. 3.11, pCGN155702) of approximately 5 kbp in size using only the 35S-1 primer in the PCR reaction resulted in more than one band, one of which was about 500 bp in size. Southern blot analysis of the PCR products demonstrated that these bands did not correspond to the BWYV coat protein gene or the *npt* II gene (results not shown). Southern blot analysis of the PCR products using the binary plasmid constructs as templates produced a 720 bp fragment for the pCGN154802, pCGN155702 (see Fig. 3.16, lanes 24-28, lanes 2-9, 11-15) constructs but no fragment for the pCGN1548 and pCGN1557 binary plasmids, as predicted (see Fig. 3.16, lane 37 and 34, respectively). Surprisingly, the BWYV coat protein gene could not be detected in any of the regenerated plants transformed with the anti-sense binary plasmid constructs (pCGN154837 and pCGN155737). However, a PCR product of 720 bp in size was obtained using the anti-sense binary plasmid constructs, pCGN154837 and pCGN155737 as controls. A 720 bp fragment was detected by Southern blot analysis of the PCR products in several plant DNA samples which included: thirteen plants treated with the *A. tumefaciens* harbouring the 155702 construct, five with the 154802 construct (including plants 154802-1 and 154802-3 which were shown previously by Southern blot analysis to possibly contain the coat protein gene) and plant 1557-7. A 630 bp fragment was revealed in the Southern blot analysis of the PCR products from the plant 1557-7 (see Fig. 3.16, lane 23), which

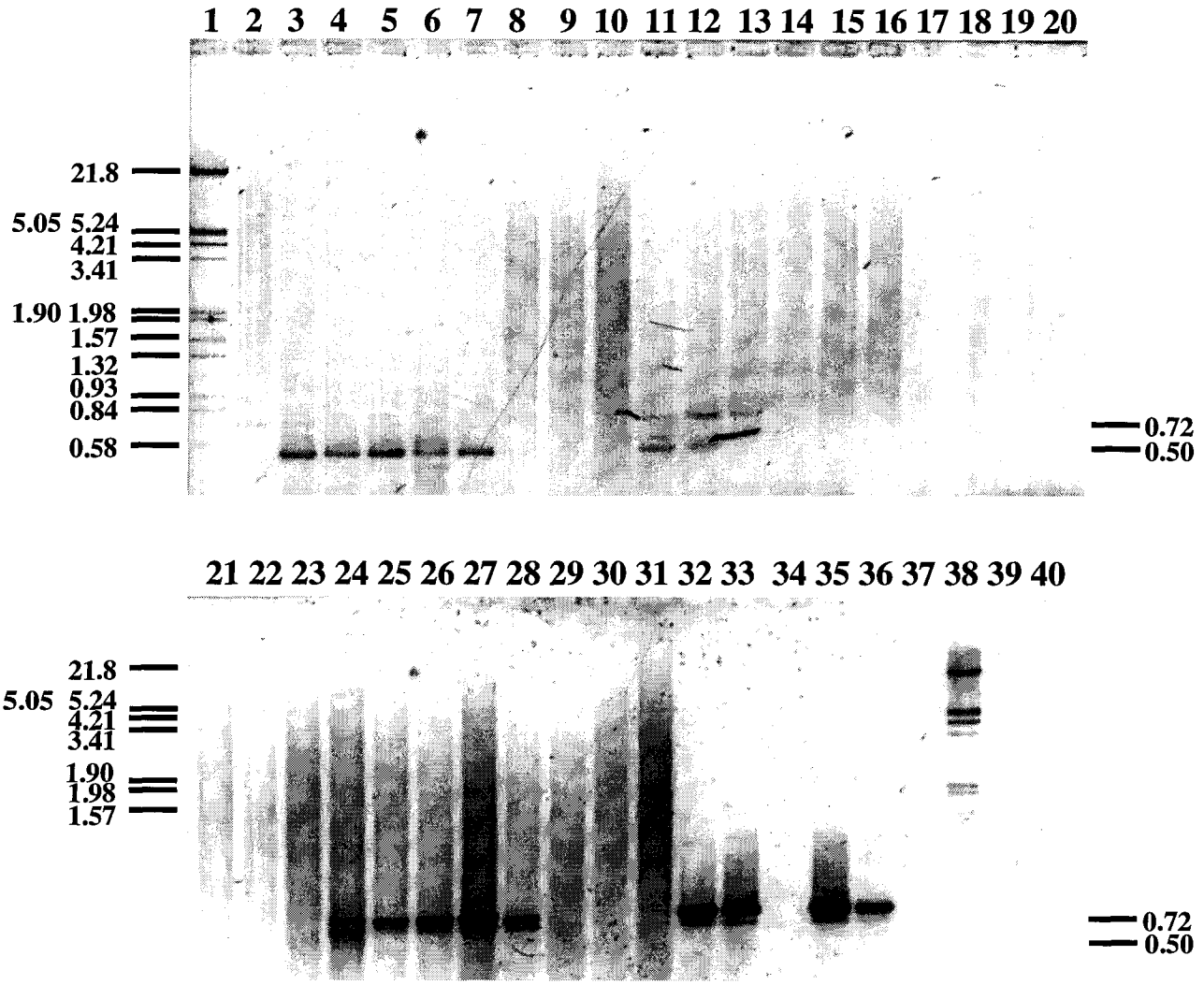


Fig. 3.17 PCR analysis of DNA extracted from regenerated plants. A PCR primer based on the CaMV 35*S* promoter sequence was used in combination with either of the PCR primers (BWYV oligonucleotide #1 and #2) for detection by the polymerase reaction of the BWYV coat protein gene (anti-sense orientation and plus-sense orientation, respectively) in DNA samples extracted from regenerated plants. Template DNA used in the PCR reactions was extracted from the following regenerated plants: 155702-1 to -15 (lanes 2-16), 155737-1 to -6 (lanes 17-22), 1557-7 (lane 23), 154802-1 to -5 (lanes 24-28), 154837-1 (lane 29) and 1548-2 to -3 (lanes 30,31). The PCR products obtained were analyzed by electrophoresis through a 1% agarose gel and stained with ethidium bromide. Templates used as controls included the binary plasmids pCGN155702, pCGN155737, pCGN1557, pCGN154802, pCGN154837 and pCGN1548 (lanes 32-37, respectively). Plant DNA extracted from uninfected *Brassica napus* cv. Westar (lane 39) and water (lane 40) were also included as the controls. In the DNA samples where the BWYV coat protein gene was not expected to be detected by PCR, all three PCR primers were used (lanes 23, 30, 31, 34, 37, 39 and 40). Lanes 1 and 38 contain molecular size markers (in kbp): Lambda DNA digested with *Eco* RI and *Hin*d III. The numbers on the right indicate the expected size of the coat protein PCR product, 720 bp and of the 500 bp non-specific band, likely a result of mis-priming using the CaMV 35S-1 primer.

was not expected to contain the BWYV coat protein gene but nevertheless hybridized to a BWYV coat protein probe when the plant DNA extracts were analyzed by Southern blotting (see Section 3.7.1). In the PCR reactions in which the DNA template was extracted from plants that were presumed to have been transformed with a binary plasmid (pCGN1548 and pCGN1557) not containing the BWYV coat protein, all three primers (BWYV oligonucleotide primer #1 and #2, and CaMV 35S-1 oligonucleotide primer) were included in the reactions. The 630 bp fragment seen in the Southern blot analysis of the PCR products from the plant 1557-7 likely resulted from the annealing of the BWYV oligonucleotide primers #1 and #2 and subsequent amplification of the BWYV coat protein. Alternatively, the annealing of the CaMV 35S-1 oligonucleotide primer and BWYV oligonucleotide primer #2 would have resulted in the amplification of a portion of the CaMV 35*S* promoter upstream of the BWYV coat protein gene as a 720 bp fragment. The results from the PCR analysis of the plant genomic DNA followed by Southern blot analysis suggests that several other plants were successfully transformed with the BWYV coat protein gene constructs: plants 155702-1 to -8, -10 to -14, 1557-7 and 154802-1 to -5. The PCR analysis did not detect the BWYV coat protein gene in any of the plants treated with the *A. tumefaciens* harbouring the construct containing the gene in the anti-sense orientation. Primers were not obtained for use in PCR to amplify the *npt* II gene from the plant DNA samples. Attempts to detect the BWYV coat protein gene by RT-PCR in RNA samples extracted from the regenerated plants were unsuccessful.

3.7.4 Evaluation of Seeds from Regenerated Plants for Kanamycin Resistance

The seeds (R1) of the primary transformants of *B. napus* cv. Westar were germinated in the presence of 50 ug/mL kanamycin (see Table 3.4). The transformed plants containing the plant selectable marker *npt* II, which confers kanamycin resistance, had green first true leaves whereas the first true leaves of plants that were not resistant to kanamycin became bleached within two weeks after germination. Two plants showed no resistance to kanamycin (untransformed *B. napus* and plant 154802-5) as indicated by the bleaching of both the first true leaves and cotyledons. Several plants showed partial resistance to kanamycin (indicated by bleaching of first true leaves but partially green cotyledons). A few plants showed only slightly better resistance as indicated by the pale green color of their first true leaves. The plants were scored for their level of resistance to kanamycin (no resistance, partial resistance and good resistance) and the segregation ratio (resistant: non-resistant plants) compared to the theoretical ratio of 3: 1; partial resistance was counted as resistance in the calculation of the segregation ratio. Of seventeen plants tested, ten plants produced seeds displaying ratios greater than 3:1, and seven had ratios less than 3:1 (see Table 3.4). Plants 154802-1 and 154802-3, which were shown previously by Southern blot analysis to possibly contain the coat protein gene but not the *npt* II gene, had ratios of 9:1 and 5:1, respectively. Surprisingly, plant 1557-7, which was shown previously by Southern blot analysis to contain not only the coat protein gene but also the *npt* II gene, had a segregation ratio of 2.2:1. Plant 154802-5, which was shown

Table 3.4 Evaluation of seeds from regenerated plants for kanamycin resistance. Seeds (R1) from the primary transformants of *Brassica napus* cv. Westar were germinated on MS media supplemented with 50 g/mL kanamycin (see Section 3.7.4). R1 seed from untransformed regenerated *B. napus* cv. Westar was also included. The plants were scored for kanamycin resistance two weeks after germination; (--) no kanamycin resistance, bleached cotyledons and first true leaves; (-+) partial kanamycin resistance, green (or partial) cotyledons and bleached first true leaves; (++) kanamycin resistance, green cotyledons and pale green first true leaves. The ratio of kanamycin resistant to non-resistant plants was calculated; a 3:1 ratio is the theoretical ratio expected. Partial kanamycin resistance was scored as resistance in determining the final ratio.

Primary Transformant	Number Germinated	Kanamycin Resistance			
		--	-+	++	ratio n:1
R1	13/20	13	0	0	0.0
155702-1	17/20	4	7	6	3.2
155702-4	17/20	3	3	11	4.7
155702-5	13/20	2	9	2	5.5
155702-6	20/20	6	11	3	2.3
155702-10	12/20	5	6	1	1.4
155702-11	14/20	2	3	9	6.0
155702-12	20/20	3	13	4	5.7
155702-13	19/20	2	5	12	8.5
155737-1	19/20	10	6	3	0.9
155737-6	19/20	4	15	0	3.8
1557-7	19/20	6	3	10	2.2
154802-1	20/20	2	8	10	9.0
154802-2	19/20	8	11	0	1.4
154802-3	18/20	3	10	5	5.0
154802-4	15/20	8	5	2	0.9
154802-5	16/20	16	0	0	0.0
154837-1	19/20	2	2	15	8.5

only by PCR analysis to contain the BWYV coat protein gene, demonstrated no resistance to kanamycin. Similarly, the progeny from untransformed *B. napus*, as expected, displayed no resistance to kanamycin. The level of resistance to kanamycin likely is a function of the number of functioning *npt* II gene integrations into the genome of the plant. Although the germination of twenty plants (seeds) is insufficient for statistical analysis, the ratios suggest that many of the plants had likely been co-transformed with the *npt* II gene. None of these plants could be recovered for further testing as they became stunted in their growth, even after being transferred to kanamycin-free medium.

3.8 Production and Screening of Monoclonal Antibodies and Polyclonal Antisera for Reactivity Against Disrupted BWYV Particles

3.8.1 Evaluation of Antibodies in Western Blot and Dot Blot Analysis

BWYV infection of *B. napus* was monitored by using a polyclonal antiserum and a monoclonal antibody in an enzyme linked immunosorbent assay (ELISA). BWYV coat protein expression in transgenic plants is expected to eventually result in the formation of coat protein subunits. Ideally, it would have been useful to screen for transgenic plants expressing BWYV coat protein subunits by ELISA. Several monoclonal antibodies (510H 1gG2a α BWYV, 43BC IgM α BWYV & PLRV, 4G12 IgM α BWYV and 26BE IgG1 α PLRV) and polyclonal antisera (rabbit α BWYV IgG

and rabbit α PLRV IgG), kindly provided by Peter Ellis (Agriculture Canada Research Station), were evaluated for the ability to react with disrupted BWYV particles. These antibodies were originally produced by injection of intact purified BWYV virions into mice and rabbits, for monoclonal antibodies and polyclonal antisera, respectively. The monoclonal antibody 510H (mouse α BWYV), the polyclonal antiserum (rabbit α BWYV) and goat IgG (goat α mouse) were used in triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) to monitor BWYV infection of *B. napus* cv. Westar (Ellis and Wieczorek, 1992).

Initially, the monoclonal antibodies were evaluated for the ability to bind BWYV coat protein under the conditions of a Western blot analysis. Various amounts (100-500 ng) of purified disrupted BWYV were electrophoresed through a 10% SDS-polyacrylamide gel and electro-blotted onto PVDF membrane (0.2 micron, BioRad). The trans-blots were incubated with the appropriate monoclonal antibody / [^{125}I]-labeled goat anti-mouse IgG, washed and autoradiographed. None of the monoclonal antibodies appeared to react with the BWYV coat protein under the denaturing conditions of the Western blot analysis. Furthermore, incubation of the polyclonal antiserum (α BWYV) / [^{125}I]-labeled goat anti-rabbit with the same trans-blots demonstrated that the polyclonal antiserum did not react with the BWYV coat protein under these conditions (results not shown).

Various amounts (100-500 ng) of intact purified BWYV virion and disrupted

BWYV particles (1% SDS, 95°C for 5 min) were spotted onto PVDF membrane, incubated with the appropriate monoclonal antibody / [^{125}I]-labeled goat anti-mouse IgG, washed and autoradiographed. All the monoclonal antibodies tested reacted with the intact purified BWYV particles on the membrane except for 26BE (α PLRV). However, none of the monoclonal antibodies reacted with the disrupted BWYV particles spotted onto the membrane. Furthermore, the polyclonal antiserum specific for BWYV also reacted with the intact purified BWYV particles spotted on the membrane but not with the disrupted BWYV particles. As expected, the polyclonal antisera specific for PLRV did not react with either intact or disrupted BWYV particles on the membrane.

Based on this analysis, the above monoclonal antibodies and polyclonal antisera would not have been useful for screening transgenic *B. napus* expressing BWYV coat protein subunits.

3.8.2 Production of Monoclonal Antibodies and Polyclonal Antisera using Disrupted BWYV Particles

Purified BWYV particle preparations were disrupted by treatment with 1% SDS and subsequent heating at 95°C for 5 min followed by dialysis against 0.1 M phosphate buffer, pH 7.0. The denatured particles were used to immunize BALB/c mice and a rabbit for the production of monoclonal antibodies and polyclonal antisera,

respectively. BALB/c mice were immunized with three injections of virus (50 ug per injection) before their spleens were harvested and used in the fusion protocol to make hybridomas (Ellis and Wieczorek, 1992). The hybridomas were screened by an indirect TAS-ELISA. Hybridoma clones secreting BWYV-specific antibodies against disrupted virus particles, but not intact virus particles could not be found. Similarly, a rabbit was immunized with five injections of virus (0.5 mg per injection). Test bleeds (30 mL) were taken after the third and fourth injections. Immunoglobulins (IgG) purified from a portion of the test bleeds were evaluated in double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) for the ability to react with intact and disrupted BWYV particles as well as possible BWYV coat protein subunits in transgenic plants. Preliminary results indicated that the purified IgGs did not react against intact BWYV particles but did react against disrupted BWYV particles (treatment with SDS or carbonate buffer, pH 9.2). The purified IgG was used in DAS-ELISA to screen for any regenerated plants that might be expressing BWYV coat protein subunits. None of the plants appeared to be expressing the BWYV coat protein at a level detectable by ELISA (results not shown).

3.9 Preliminary Evaluation of BWYV Resistance in Transgenic *Brassica napus* cv.Westar

The seeds (R1) from the primary transformants of *B. napus* cv. Westar (18 plants from 154802-3, 14 plants from 155737-6, 14 plants from 1557-7) and the seeds

(R1) from untransformed regenerated plants were germinated and placed in pots in the greenhouse (16 hr light, 21°C / 8 hr darkness, 16°C). Limited space dictated that a limited number of transgenic lines be evaluated. The progeny of plants 154802-3 and 1557-7 were evaluated because they were previously shown by Southern blot and PCR analysis to contain the BWYV coat protein gene. Thus, plants 154802-3 and 1557-7 were expected to show the best levels of resistance to infection by BWYV. Plant 155737-6 could not be demonstrated in Southern blot analysis or PCR analysis to contain the BWYV coat protein. However, germination of the progeny from this plant showed that they possessed good resistance to kanamycin. Thus, plant 155737-6 was not expected to show any resistance to infection by BWYV. After 2-3 weeks, when the first true leaves were 4-5 cm in length, 20-40 viruliferous green peach aphids (*Myzus persicae*) were placed on the plants for an inoculation access period of 72 hours. Prior to the inoculation, the aphids were allowed at least 48 hr access to BWYV-infected plants. *Physalis pubescens* L., an indicator plant, was also inoculated at the same time. After 5-6 weeks, leaf samples were taken from each plant and tested by TAS-ELISA for BWYV infection. Neither the inoculated transgenic nor the untransformed *B. napus* cv. Westar nor the indicator plants showed any of the typical symptoms of BWYV infection. However, all plants were infected with a high titre of BWYV as determined by TAS-ELISA (results not shown).

Discussion

The beet western yellows virus (BWYV) coat protein gene was successfully introduced into the genome of *Brassica napus* cv. Westar via the *Agrobacterium tumefaciens* Ti plasmid mediated gene transfer. A summary of the integration and expression analysis of the regenerated plants is provided in Table 4.1. Southern blot analysis of *Hin*d III digested plant DNA indicated that the BWYV coat protein gene was present in the genome of three plants (154802-1, 154802-3 and 1557-7). However, Southern blot analysis of *Eco* RI digested plant DNA showed that the *npt* II gene was present only in plants 1557-7 and 155737-5 but not 154802-1 and 154802-3. Furthermore, the blots of the same *Eco* RI digested plant DNA showed that the BWYV coat protein gene was present in the genome of plants 1557-7 and 154802-3. PCR analysis of the same plant genomic DNA followed by Southern blot analysis of the PCR products indicated that several plants contained the BWYV coat protein gene in the plant genome (plants 155702-1 to -8, -10 to -14, 1557-7 and 154802-1 to -5). No RNA transcripts of either the BWYV coat protein or of the *npt* II gene product could be detected in Northern blots but germination of the seeds from the primary transformants on kanamycin indicated that the *npt* II gene product, which confers kanamycin resistance, is functional in many of the plants. However, preliminary evaluation of the progeny of three promising lines, demonstrated to have the BWYV coat protein gene integrated into their genomes, did not find significant levels of

Table 4.1 Summary of integration and expression analysis of regenerated *Brassica napus* cv. Westar after treatment of cotyledonary explants using the plant transformation procedure (Moloney et al., 1989). (n/s) No signal was detected for the indicated gene, (???) the plant is highly suspect for the presence of the indicated gene, (n/t) the sample was not tested, (n/a) not applicable.

Date plant placed into greenhouse	Construct	Southern blot analysis			Northern blot analysis		PCR	Germination analysis	
		Eco RI digested plant DNA		Hind III digested				kanamycin resitance	
		nptII gene	BWYV CP gene	BWYV CP	npt II gene	BWYV CP	BWYV CP gene	#	ratio n:1
April 1/92	155737-1	n/s	n/s	n/s	n/s	n/s	n/s	9/19	0.9
April 30/92	155702-1	n/s	n/s	n/s	n/s	n/s	Yes	13/17	3.2
	155737-2	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	155737-3	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1557-1	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1557-2	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1557-3	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1557-4	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
May 1/92	155702-2	n/s	n/s	n/s	n/s	n/s	Yes	n/t	n/t
June 2/92	155702-3	n/s	n/s	n/s	n/s	n/s	Yes	n/t	n/t
	155702-4	n/s	n/s	n/s	n/s	n/s	Yes	14/17	4.7
	155702-5	n/s	n/s	n/s	n/s	n/s	Yes	11/13	5.5
	155702-6	n/s	n/s	n/s	n/s	n/s	Yes	14/20	2.3
	155737-4	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
July 2/92	155702-7	n/s	n/s	n/s	n/s	n/s	Yes	n/t	n/t
	155702-8	n/s	n/s	n/s	n/s	n/s	Yes	n/t	n/t
	155702-9	n/s	n/s	n/s	n/a	n/a	n/s	n/a	n/a
	155702-10	n/s	n/s	n/s	n/s	n/s	Yes	7/12	1.4
	155702-11	n/s	n/s	n/s	n/s	n/s	Yes	12/14	6.0
	155702-12	n/s	n/s	n/s	n/s	n/s	Yes	12/14	6.0
	155702-13	n/s	n/s	n/s	n/s	n/s	Yes	17/19	8.5

Table 4.1 (cont'd)

Date plant placed into greenhouse	Construct	Southern blot analysis			Northern blot analysis		PCR	Germination analysis	
		Eco RI digested plant DNA		Hind III digested				kanamycin resitance	
		nptII gene	BWYV CP gene	BWYV CP	npt II gene	BWYV CP	BWYV CP gene	#	ratio n:1
July 2/92	155702-14	n/s	n/s	n/s	n/s	n/s	Yes	n/t	n/t
	155702-15	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	155737-6	n/s	n/s	n/s	n/s	n/s	n/s	15/19	3.8
	1557-5	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1557-6	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1557-7	Yes	Yes	Yes	n/s	n/s	Yes	13/19	3.2
	1557-8	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	154802-1	n/s	n/s	Yes	n/s	n/s	Yes	18/20	9.0
	154802-2	n/s	n/s	n/s	n/s	n/s	Yes	11/19	1.4
	154802-3	???	???	Yes	n/s	n/s	Yes	15/18	5.0
	154802-4	n/s	n/s	n/s	n/s	n/s	Yes	7/15	0.9
	154802-5	n/s	n/s	n/s	n/s	n/s	Yes	0/16	0.0
	154837-1	???	n/s	n/s	n/s	n/s	n/s	17/20	5.7
	1548-1	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1548-2	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	1548-3	n/s	n/s	n/s	n/s	n/s	n/s	n/t	n/t
	untreated	n/s	n/s	n/s	n/s	n/s	n/s	0/13	0.0

resistance when these plants were inoculated with the homologous virus using the aphid vector, *Myzus persicae* (Sulz.).

4.1 Transformation of *Brassica napus* cv. Westar

The plant transformation procedure used was that described by Moloney *et al.* (1989) for the transformation of *Brassica napus* cv. Westar utilizing cotyledonary explants. The regeneration efficiency of the cotyledonary explants, after inoculation with the *A. tumefaciens*, ranged from 0.8-3.6% as compared to 17-80% in the uninoculated cotyledonary explants. Thirty-eight regenerated plants were recovered from 4670 cotyledonary explants which had been inoculated with *A. tumefaciens*. The regeneration efficiency observed was considerably less than that reported (55%) by Moloney *et al.* (1989) but compared favorably with previous work described using floral stem 'thin cell layer' (2% efficiency, Charest *et al.*, 1988), hypocotyls (2.5% efficiency, Radke *et al.*, 1988) and longitudinal stem sections (10% efficiency, Pua *et al.*, 1987). Two other groups have observed low regeneration efficiency (Calgene Inc., California and Plant Biotechnology Institue, Saskatoon; personal communication) using this same plant transformation procedure. Additional difficulties with the plant transformation procedure included: (1) contamination of explants when using high inocula of *A. tumefaciens*, even under the selection of the antibiotic, carbenicillin and (2) the apparently high number of regenerated plants which escaped selection by kanamycin. The contamination was greatest in the third (July 1992) and fourth

(September 1992) attempts to transform *B. napus* cv. Westar. Although the regeneration efficiency of shoots remained low for the third and fourth attempts, the plants that did regenerate were soon overcome by the rapidly growing *A. tumefaciens* and died. Of the thirty eight plants regenerated during the first two plant transformation attempts, it became apparent from germination studies that 33-40% of the putative transgenic plants possessed little or no resistance to kanamycin; even though the regenerated plants were grown in the selection of kanamycin. Several modifications to the plant transformation procedure (kindly suggested by S. Radke, Calgene and G.H. van Rooijen, University of Calgary) may address the difficulties observed with this procedure. (1) Reducing the initial inoculum of *A. tumefaciens*, reducing the co-cultivation period of the *A. tumefaciens* with the cotyledonary explants from 72 hours to 24 hours and using cefotaxime in combination with the carbenicillin might reduce the amount of tissue necrosis and hopefully increase the regeneration efficiency of the plant transformation procedure. The *B. napus* cv. Westar cotyledonary explants appeared to be sensitive to high levels of *A. tumefaciens* inoculum. A large inoculum resulted in early tissue necrosis, especially along the exposed cut ends of the explants. Eventually, the high concentrations of *A. tumefaciens* overcame the antibiotic selection with carbenicillin and killed any shoots that managed to regenerate into plants. (2) Although exposure to kanamycin was found to increase the time required for the formation of roots (Moloney *et al.*, 1989), the use of kanamycin throughout the procedure should reduce the possibility that plants that are not kanamycin resistant, and therefore not transformed, will survive.

The plant transformation procedure described by Moloney *et al.* (1989) uses kanamycin only up to the shoot stage. Since the polarity of T-DNA gene transfer occurs from the right border to the left border in the Ti plasmid (Rubin, 1986), plants transformed with the *npt* II gene presumably would have been co-transformed with the BWYV coat protein using the binary plasmid constructs described in Section 3.4.

4.2 Integration Analysis of Regenerated *Brassica napus* cv. Westar

The genomic DNA extracted from the regenerated plants was analyzed by Southern blot analysis and PCR analysis (see Table 4.1). Southern blot analysis of *Hind* III digested genomic DNA, using a random primed, [^{32}P]-labeled probe corresponding to the BWYV coat protein gene, indicated that the BWYV coat protein gene was present in the genome of three plants (154802-1, 154802-3 and 1557-7). However, Southern blot analysis of *Eco* RI digested genomic DNA, using a random primed, [^{32}P]-labeled probe corresponding to the *npt* II gene, showed that the *npt* II gene was present only in the genome of plants 1557-7 and 155737-5. Surprisingly, the *npt* II gene was not detected in the genome of plants 154802-1 and 154802-3. Probing of the same Southern blots, after stripping the *npt* II cDNA probe from the blots, with random primed, [^{32}P]-labeled DNA corresponding to the BWYV coat protein gene indicated that the BWYV coat protein gene was present in the genome of plant 1557-7 and 154802-3. But the BWYV coat protein gene could not be detected in the genome of plants 154802-1 and 155737-5 after digestion of their DNA with

Eco RI. The reason that the plant transformed with construct 154802-1 hybridized to the coat protein probe when the plant DNA was digested with *Hin*d III but not with *Eco* RI is not understood at this time. It is possible that the coat protein gene is indeed present but could not be detected in the Southern blot analysis due to problems with the particular plant DNA sample (possibly degradation); especially since the PCR analysis indicated the presence of the coat protein gene. The lack of hybridization to the *npt* II probe using the same *Eco* RI digested DNA could be explained similarly. Indeed the result from the germination analysis of R1 seed would support the finding that the original plant was transformed at least with the *npt* II gene since the segregation ratio was 9:1. Similarly, germination analysis of R1 seeds from plants transformed with 154802-3 would suggest the presence of the *npt* II gene (see Table 4.1).

The genome size of *Brassica napus* has been reported to be 2.3-2.56 pg/2C or 1129-1235 Mbp/C (Arumuganathan and Earle, 1991; where 2C represents the diploid genome and 1C the haploid genome). Assuming that only a single copy of the BWYV coat protein gene is present in each genome, 100 ug of plant genomic DNA would contain *ca.* 25 pg of BWYV coat protein DNA (Rogers and Bendich, 1988). Twenty-five picograms of plasmid DNA gave very strong signals in the Southern blots, so the lack of a hybridization signal in the plant DNA samples should mean that these plants were not transformed. However, these conclusions are difficult to draw in view of the variable intensity of signal obtained with control plasmid DNA which was

intended to be 25 pg / lane (see Fig. 3.13, lanes 13, 14, 16 and 17). The BWYV coat protein gene was detected in several other plants (plants 155702-1 to -8, -10 to -14, 1557-7 and 154802-1 to -5) by PCR analysis of the genomic DNA followed by Southern blot analysis. PCR primers for the detection of the *npt* II gene were not available. Since the polarity of T-DNA transfer is from the right border to the left border of the Ti plasmid (Rubin, 1986), the BWYV coat protein gene would have been transferred before the *npt* II gene in the binary plasmid constructs was used (see Section 3.4). The possibility arises that a plant can be transformed with the BWYV coat protein gene but not the *npt* II gene (see Section 3.4). This may be the case for several plants (1548002-2, -4,-5 and 155702-10) which were shown by PCR analysis to contain the BWYV coat protein but showed little or no resistance to kanamycin in germination studies, as evidenced by the low segregation ratios (see Table 4.1). Truncated or rearranged integration has been previously reported in *Brassica napus* transformants (Radke *et al.*, 1988) and in *Nicotiana tabacum* (Van Lijsbettens *et al.*, 1986).

4.3 Expression analysis of regenerated *Brassica napus* cv. Westar

The expression of the BWYV coat protein and the *npt* II gene product were evaluated in Northern blots, by a functional bioassay for kanamycin resistance and by ELISA. Northern blot analysis of the RNA extracted from the regenerated plants could not detect either BWYV coat protein transcripts or *npt* II transcripts. Previous

work with transgenic potato transformed with the PLRV coat protein gene indicated that PLRV coat protein RNA transcripts could be detected in a total leaf RNA extract only after a poly(A) RNA enrichment had been performed (Kawchuck *et al.*, 1990). Germination of the *B. napus* R1 seeds from the primary transformants on kanamycin-containing media indicated that the *npt* II gene product was functional in many of the transformed plants (see Section 3.7.4) suggesting that *npt* II transcripts are present in the plant but were not detected in the blotting experiments described in this thesis. It is possible that many of the plants which showed kanamycin resistance represent "escapes" however, the germination analysis from seeds derived from the primary transformants indicated that the kanamycin resistant phenotype segregated as a dominant gene (~ 3:1 ratio) and therefore these plants likely do not represent kanamycin "escapes". BWYV coat protein subunits were not detected by DAS-ELISA using a polyclonal antisera that was produced using BWYV coat protein subunits. Although the CaMV 35*S* promoter has been known to confer high levels of expression in a variety of plants cells, the expression levels of the BWYV coat protein and the *npt* II gene product in the primary transformants appeared to be low or not detectable. In transgenic potatoes, containing the PLRV coat protein chimeric gene under the control of the duplicated CaMV 35*S* promoter, transcription levels of the PLRV coat protein gene were high but little or no coat protein was detected (Kawchuk *et al.*, 1990). Inoculation of the transgenic potato plants with PLRV resulted in low virus titers that remained low or decreased indicating sustained resistance. Similar resistance to PLRV infection was found with plants transformed with the PLRV coat protein gene in anti-

sense orientation which suggests that resistance of transgenic potatoes against PLRV may be mediated by the coat protein gene transcript. Preliminary evaluation of the progeny of two promising lines (154802-3 and 1557-7), which were shown by Southern blot analysis and PCR analysis to contain the BWYV coat protein gene in the plant genome, did not find significant levels of resistance when these plants were inoculated with the homologous virus using the aphid vector, *Myzus persicae* (Sulz.).

4.4 Future outlook

The ability to transform *Brassica napus* cv. Westar has proven useful for overall improvement to the quality of the crop and for the study of plant, bacterial and viral genes. Significant improvements in the quality of the oil and protein content of *B. napus* seeds have been obtained by the introduction of the stearoyl-acyl carrier protein desaturase gene in the antisense orientation (Knutzon *et al.*, 1992) and a methionine-rich seed protein from a Brazil nut (Altenbach *et al.*, 1992). The beet western yellows virus (BWYV) coat protein gene was successfully introduced into the genome of *Brassica napus* cv. Westar via the *Agrobacterium tumefaciens* Ti plasmid mediated gene transfer. Regeneration efficiency of *B. napus* cv. Westar using the plant transformation procedure as described by Moloney *et al.* (1989) was lower than reported. But Southern blot analysis and PCR analysis indicate, although not conclusively, that the coat protein gene has been integrated into the plant genome. It is not understood at this time the reason why BWYV coat protein RNA transcripts or

BWYV coat protein subunits were not found. But the lack of BWYV coat protein RNA transcripts and of coat protein subunits could explain why *B. napus* cv. Westar transformed with the BWYV coat protein gene showed no resistance to infection by BWYV when inoculated with infected *Myzus persicae* (Sulz.). The usefulness of the plant transformation procedure described by Moloney *et al.* (1989) will depend on improvement of the overall regeneration and transformation efficiency and development of a procedure for other *Brassicaceae* cultivars such as *Brassica juncea.* Several other genes besides the coat protein gene have proven useful for conferring viral resistance in transgenic plants; these include a portion of the viral replicase non-structural gene of TMV in tobacco (Golemboski *et al.*, 1990), viral satellite RNA from CMV in tobacco (Baulcombe *et al.*, 1986), virus specific antibodies (Baulcombe, 1986) and ribozymes in tobacco (Haseloff and Gerlach, 1988). The commercial success of pathogen resistant transgenic plants will depend on the identification of plant resistance genes rather than foreign genes, the development of plant transformation selectable markers found naturally in plants (especially in transgenic plant products that are for human consumption) and the alleviation of fears towards the release of genetically altered plants.

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