INTERACTION OF TURNIP MOSAIC VIRUS (TuMV) WITH MEMBERS OF THE *BRASSICACEAE*

BY

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University of Birmingham

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ABSTRACT

Attempts to identify *Turnip mosaic virus* (TuMV) resistance in ecotypes of *Arabidopsis thaliana* proved unsuccessful, although methods for successful inoculation and assessment of TuMV infection were developed.

Thirty-three sources of resistance to TuMV have been identified in *Brassica rapa* and *B. napus*. Fourteen lines were resistant to TuMV isolates representing the most prevalent TuMV pathotypes, 1, 3 and 4. Lines with novel specificites were also identified. For the first time, a source of resistance to pathotype 4 was identified in *B. napus* line 22S.

The genetic basis of resistance to TuMV was determined for seven *Brassica* lines. For six lines, crosses were made to susceptible genotypes and segregation of the resistance phenotype was followed in the backcross progeny. For the remaining line, Jong Bai No. 1, segregation of the resistance phenotype was followed in an F_2 population. Monogenic, dominant resistance models were proposed for *B. napus* lines 22S, 165 and *B. rapa* line Jong Bai No. 2. Recessive monogenic and multigenic models were proposed for the remaining *B. rapa* lines.

The allele conferring novel resistance in line 22S, *TuRB03*, was mapped to the lower region of *B. napus* linkage group N6, using amplified fragment length polymorphism and microsatellite bulk segregant analysis, to the same interval as *TuRB01* and *TuRB01b*. The allele conferring novel resistance in line Jong Bai No. 2 was roughly mapped to the upper region of linkage group N6. The alleles conferring resistance to pathotypes 1 and 3 in line 165 were loosely mapped to N6 and N8 (or N18) respectively.

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ABBREVIATIONS

VIRUS ABBREVIATIONS

BCMV	Bean common mosaic virus
PPV	Plum pox virus
PSbMV	Pea seed-borne mosaic virus
PVA	Potato virus A
PVX	Potato virus X
PVY	Potato virus Y
TEV	Tobacco etch virus
TMV	Tobacco mosaic virus
TuMV	Turnip mosaic virus
TVMV	Tobacco vein mottling virus

COMMON ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
A-genome	*Brassica rapa* genome
B_1	First backcross generation
BSA	Bovine serum albumin
B-SA	Bulk segregant analysis
CDN 1	TuMV isolate (type member, pathotype 4)
C-genome	*Brassica oleracea* genome
CHN 5	TuMV isolate (pathotype 3)
CZE 1	TuMV isolate (type member, pathotype 3)
CI	Cylindrical inclusion bodies
ELISA	Enzyme linked immunosorbent assay
GFP	Jellyfish green fluorescent protein
GUS	β-glucuronidase
ISEM	Immunosorbent electron microscopy
LRR	Leucine rich repeats

LZ	Leucine zipper
MAS	Marker assisted selection
NBS	Nucleotide binding site
PBS-T	Phosphate buffered saline containing 0.5% Tween-20
R1	Line 22S CDN 1 resistant bulk 1
R2	Line 22S CDN 1 resistant bulk 2
R3	Line 165 UK 1 resistant bulk
R4	Line 165 CZE 1 resistant bulk 1
R5	Line 165 CZE 1 resistant bulk 2
R6	Line Jong Bai No. 2 UK 1 resistant bulk 1
R7	Line Jong Bai No. 2 UK 1 resistant bulk 2
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
S1	Line 22S CDN 1 susceptible bulk 1
S2	Line 22S CDN 1 susceptible bulk 2
S3	Line 165 UK 1 susceptible bulk
S4	Line 165 CZE 1 susceptible bulk 1
S5	Line 165 CZE 1 susceptible bulk 2
S6	Line Jong Bai No. 2 UK 1 susceptible bulk 1
S7	Line Jong Bai No. 2 UK 1 susceptible bulk 2
TGM	*Brassica juncea* cultivar Tendergreen
UK 1	TuMV isolate (type member, pathotype 1)

CHAPTER 1

GENERAL INTRODUCTION

1.1 *BRASSICACEAE*

The *Brassicaceae* (formerly *Cruciferae*) are morphologically diverse and economically valuable. The family is made up of roughly 350 genera and approximately 3,500 species. Included in the *Brassicaceae* are vegetables (including members of the *Brassica* genus), ornamental species, condiment sources, annual wildflowers, weeds (including *Arabidopsis thaliana*) and the basis for both edible and industrial oils. The family members' flowers are in the form of a cross hence the previous family name *Cruciferae*, with four petals, usually white, yellow, or lavender, and an equal number of sepals. There are four long and two short stamens and a two-chambered ovary positioned above the other flower parts. The seeds are produced in podlike fruits.

1.1.1 *Brassica* spp.

The genus *Brassica* is an economically important group of plants (Paterson *et al.*, 2000). Members of this genus have been cultivated as forage and vegetable crops for centuries. The first description of them was by Pliny during the Roman occupation of Great Britain (Fussell, 1955). More recently brassicas have been cultivated as agronomically important vegetable, forage, oilseed and condiment crops throughout the world.

There are six species in the *Brassica* genus: three diploid species and three amphidiploid species. The genetic relationship of these *Brassica* species was proposed by U (1935) and is shown in Figure 1.1. Based on cytological evidence, U (1935) proposed that pairwise interspecies hybridisations occurred between the diploid species, *B. rapa* (A genome, 2n=20), *B. nigra* (B genome, 2n=16) and *B.*

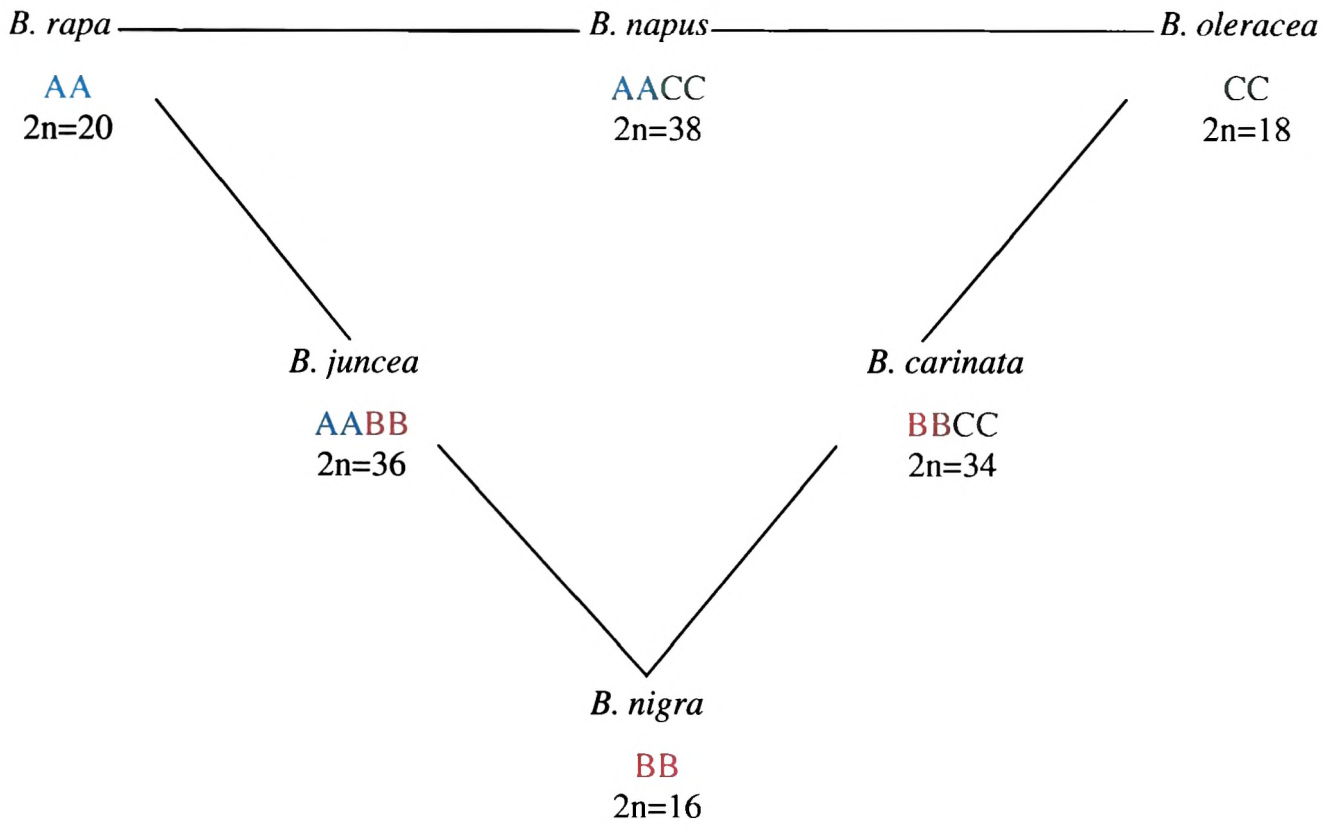


Figure 1.1 "Triangle of U" showing genetic relationships of cultivated *Brassica* species (*Brassica rapa*, *B. napus, B. oleracea*, *B. carinata*, *B. nigra* and *B. juncea*) (U, 1935).

oleracea (C genome, 2n=18) to produce the amphidiploid species, *B. juncea* (AABB, 2n=36), *B. napus* (AACC, 2n=38) and *B. carinata* (BBCC, 2n=34).

B. nigra (black mustard), *B. juncea* (brown mustard) and *B. carinata* (Ethiopian mustard) are cultivated as condiment crops and for the production of spices. The *Brassica* species that are most commonly cultivated are the amphidiploid *B. napus* and diploid *B. rapa* and *B. oleracea* species (Bunting, 1986).

B. napus and *B. rapa* are cultivated mainly as oilseed crops in Europe, providing rapeseed oil for both industrial (lubricant) and culinary purposes (in the production of margarine) and as a constituent of condiments in India, where it is also used as bath and hair oil (McNaughton, 1995). They are also used as meal for animal feed, but high glucosinolate content was a problem because it caused a bitter taste and could cause thyroid problems (Thompson & Hughes, 1986). Low glucosinolate varieties have been bred to overcome this problem. Oilseed rape is of particular economic importance in the major rapeseed growing regions of the world: China (7.8 million hectares), India (6.3 million hectares), Europe (4.9 million hectares) and Canada (4.8 million hectares) (Anon, 2001). Approximately 40 million metric tonnes of *Brassica* rapeseed was produced in 2000 (Anon, 2001).

B. napus and *B. rapa* are also grown as vegetable and fodder crops. *B. napus* has traditionally been grown as a fodder crop for livestock but is now cultivated for human consumption as swede in the UK (McNaughton, 1995) and North America (Shattuck & Stobbs, 1987; McNaughton, 1995). In Europe and New Zealand, *B. rapa* is an important forage crop for sheep and cattle (McNaughton, 1995). In China and Japan *B. rapa* is grown as important leafy vegetable crops for human consumption (Niu *et al.*, 1983; McNaughton, 1995; Suh *et al.*, 1996). Chinese cabbage and Pak choi are grown extensively in Korea, Japan (Suh *et al.*, 1996) and China (Niu *et al.*, 1983). Chinese cabbages are also grown as speciality crops in the USA (Niu *et al.*, 1983; McNaughton, 1995) and Europe (McNaughton, 1995).

The diploid species *B. oleracea* represent a large proportion of European vegetable consumption. It is a highly polymorphic species encompassing a wide range of important vegetable and fodder crops, for example cabbage, Brussels sprouts, broccoli, calabrese, cauliflower, kohlrabi and kale (Hodgkin, 1995).

1.1.2 *Arabidopsis thaliana*

Arabidopsis thaliana is a member of the family *Brassicaceae* and is closely related to the *Brassica* genus (Hedge, 1976; Meyerowitz & Prewitt, 1985). The exact relationship between *Brassica* species and *A. thaliana* is unclear, but several studies show conserved regions between the genomes of both species, suggesting they share common ancestry (Kowalski *et al.*, 1994; Lagercrantz *et al.*, 1996; Cavell *et al.*, 1998).

The *A. thaliana* plant is well suited to classical genetic work: it has a rapid generation time (five weeks); it can produce 10,000 seeds per plant; and its small size means many plants can be grown in a small area (Meyerowitz & Pruitt, 1985). It is self-fertile which allows new mutations to be made homozygous with minimal effort. It has the smallest known genome among higher plants, with a haploid nuclear genome size of approx. 70,000kb (Arumuganathan & Earle, 1991). The genome also has a very low content of repeated sequences.

The combined advantages listed above have made *A. thaliana* a useful model for plant-pathogen interaction studies. It has been used in studies on *Pseudomonas syringae* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995), *Peronospora parasitica* (Parker, *et al.*, 1993; Parker *et al.*, 1997; Bittner-Eddy *et al.*, 1999; Bittner-Eddy *et al.*, 2000) and *Tobacco etch virus* (TEV) (Mahajan *et al.*, 1998; Chisholm *et al.*, 2000; Whitham *et al.*, 2000).

1.2 THE POTYVIRUS GENUS

The potyvirus genus is the largest of the six genera in the *Potyviridae* family (Table 1.1). The *Potyviridae* family is the largest plant virus family, containing over 30% of all known plant viruses. Members of the *Potyviridae* are of great economic importance because they are distributed world-wide and infect a wide range of agricultural, pasture, horticultural and ornamental crops causing losses of yield and quality (Ward & Shukla, 1991). A major taxonomic criterion of potyviruses is the formation of cytoplasmic inclusion bodies (CI) in infected cells (Edwardson, 1974; Fenner, 1976; Ward & Shukla, 1991), seen as pinwheel structures in transverse section (Figure 1.2).

1.2.1 Genome Structure and Organisation

All potyviruses have flexuous, rod-shaped virions, 680 to 900nm long and 11-15nm wide (Figure 1.3), made up of about 2000 units of a single protein which encapsidates a positive strand ssRNA genome approx. 10,000 nucleotides long (Dougherty & Carrington, 1988).

The genome of all potyviruses has a 5' terminal genome-linked protein (VPg) and a 3' poly (A) tail. It contains one long open reading frame, which is translated into a large polyprotein (between 340k and 368k) which is subsequently cleaved into ten mature proteins. Sequences of a number of aphid transmitted monopartite potyvirus genomes have shown that all of these viruses possess a conserved gene order (Shukla *et al.*, 1991). The ten gene products are (from 5' to 3'): first protein (P1), the helper component protein/proteinase (HC), third protein (P3), a putative 6K peptide ($6K_1$), the cylindrical inclusion protein (CI), a second 6K peptide ($6K_2$), the nuclear inclusion protein "a" (NIa; VPg and proteinase), the nuclear inclusion protein "b" (NIb) and the capsid protein (CP) (Figure 1.4) (reviewed by Riechmann *et al.*, 1992).

Table 1.1 Genera of the *Potyviridae* family (from Shukla *et al.*, 1994; Mayo & Brunt, 2001)

Genus	No. of members (Approx.)	Type member
Potyvirus	180	Potato virus Y (PVY)
Rymovirus	9	Ryegrass mosaic virus (RGMV)
Bymovirus	5	Barley yellow mosaic virus (BaYMV)
Macluravirus	2	Maclura mosaic virus (MacMV)
Ipomovirus	2	Sweet potato mild mottle virus (SPMMV)
Tritimovirus	1	Wheat streak mosaic virus (WSMV)

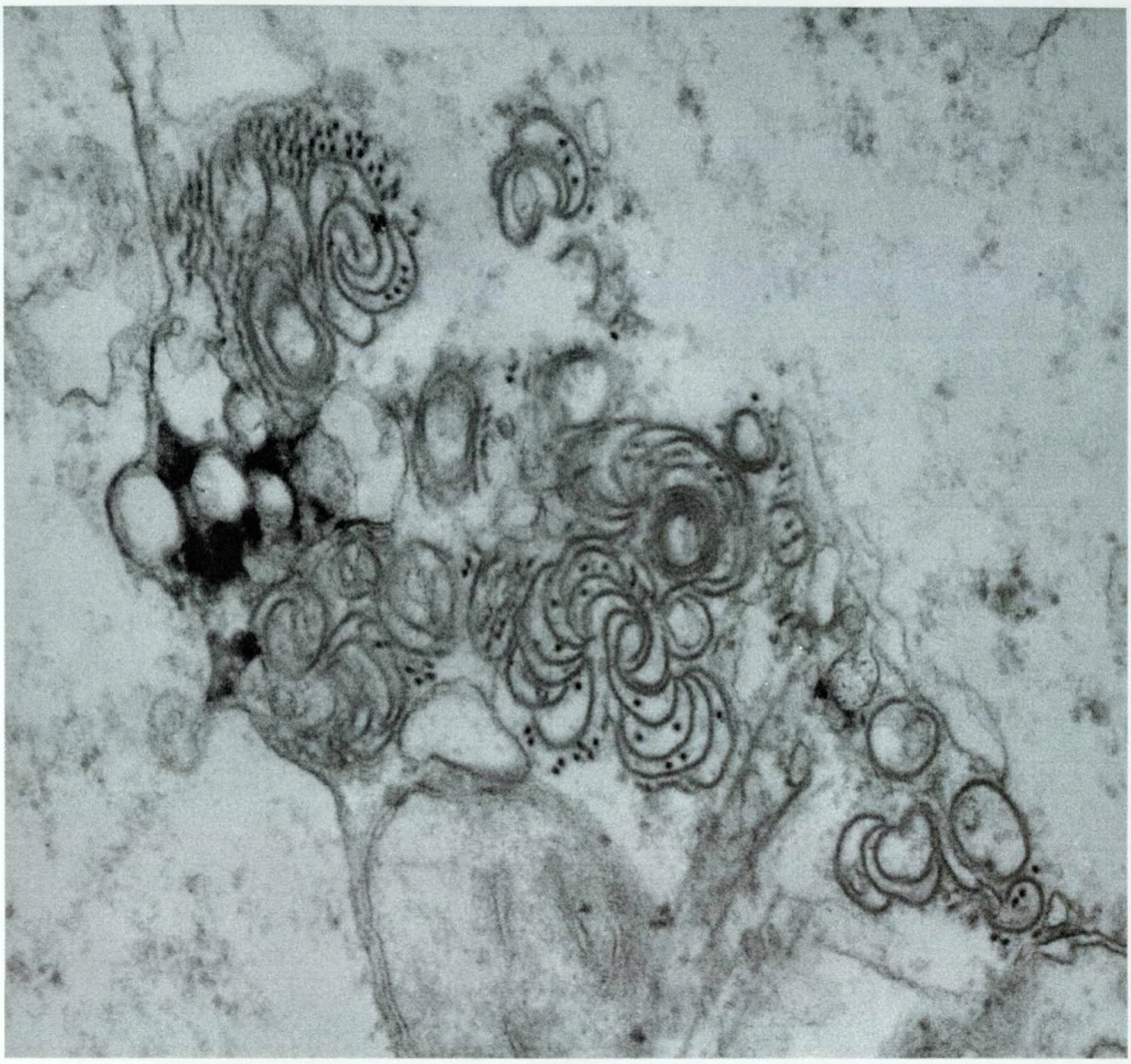


Figure 1.2 Electron micrograph transverse section of cylindrical inclusion bodies, seen as "pinwheels", in a cell infected by turnip mosaic potyvirus. Picture courtesy of C. Clay, HRI, Wellesbourne.

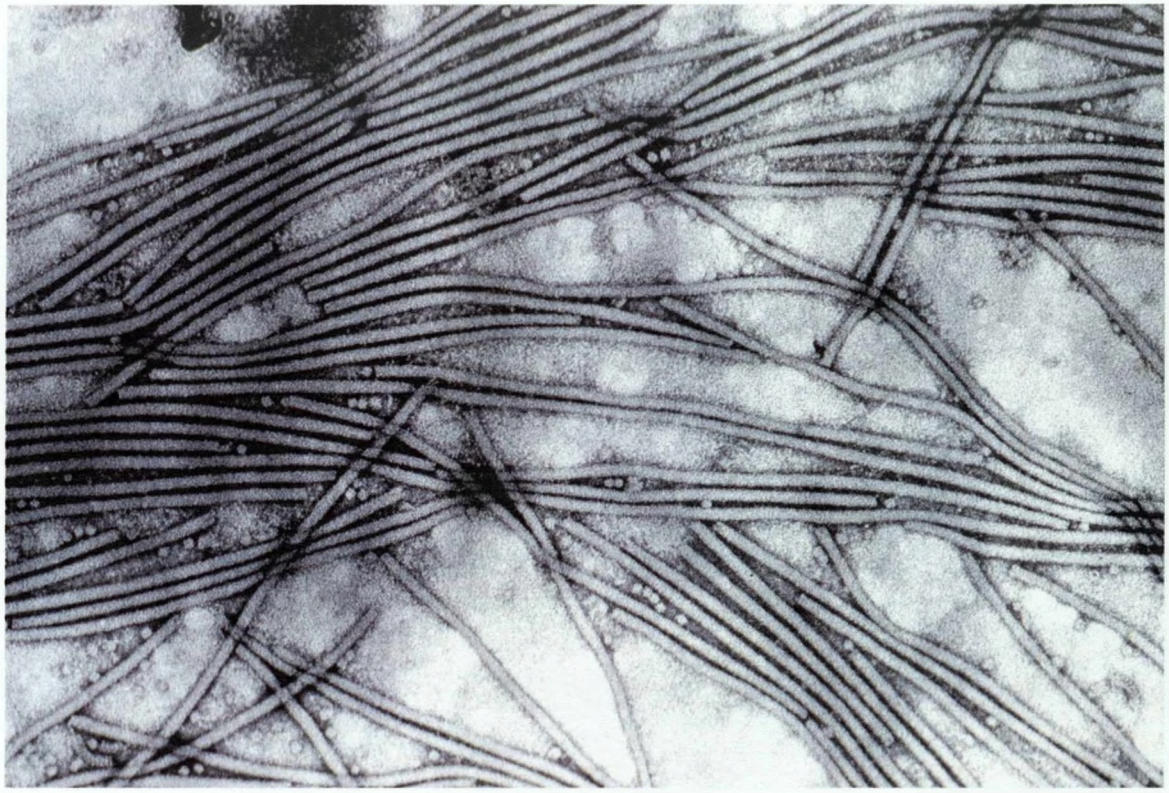


Figure 1.3 Electron micrograph of turnip mosaic potyvirus showing the typical formation of flexuous rod particles. Courtesy of C. Clay, HRI Wellesbourne.

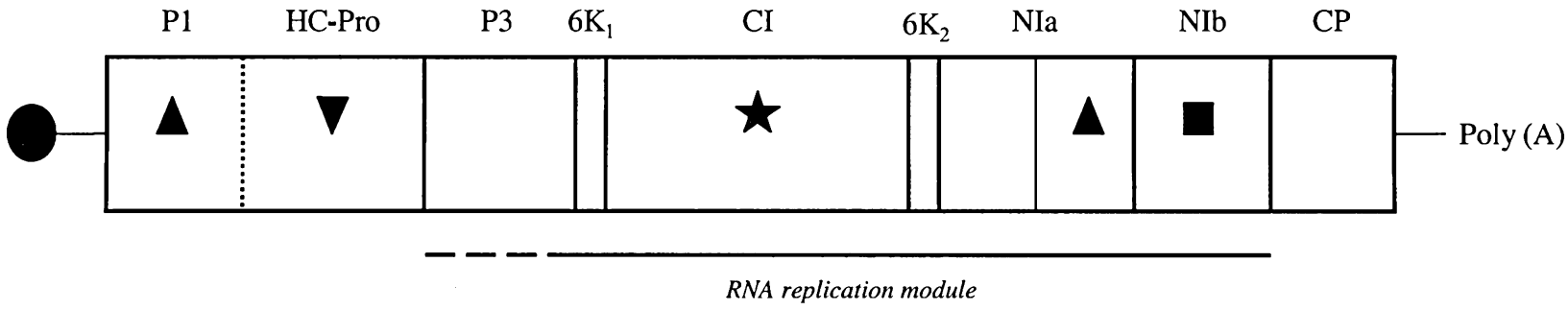


Figure 1.4 Schematic representation of the potyvirus RNA genome. The open reading frame is represented as an open bar and the VPg is represented as a black circle; vertical solid lines indicate defined cleavage sites of the polyprotein and dotted lines represent sites whose sequence and exact location is not known. Characteristic amino acid features are indicated: ▲ , serine-type protease domain; ▼ , cysteine protease domain; ★, nucleotide binding motif; ■ , polymerase domain. The name of each protein product is indicated above the corresponding cistron. RNA replication modules (composed of the cistrons that encode proteins that have been shown or suggested to be involved in RNA replication) are underlined (from Reichmann *et al.*, 1992).

1.2.2 Potyvirus Lifecycle

Viral infection of a plant begins with entry of the virus into the cytoplasm of the plant cell by overcoming the plants physical barriers, e.g. thick, waxy cuticles, either through a wound or by a vector. Once into the cell cytoplasm the virus uncoats, releasing the genomic RNA which is then amplified. There are two processes in genome amplification: RNA translation and RNA replication. To achieve both these events potyviruses use host cell resources such as amino acids, ribosomes, enzymes and nucleotides. For successful systemic infection the replicated virus must move from cell-to-cell through plasmodesmata then long-distance throughout the plant via the phloem.

The current knowledge of the function of the potyviral proteins in the virus lifecycle is incomplete. Roles of the proteins have been proposed based on sequence homology comparisons with other viral proteins of known function. The generation of full-length infectious clones and the study of protein-protein interactions through yeast two hybrid systems have revealed that most potyviral proteins are multifunctional (Table 1.2) (reviewed by Riechmann *et al.*, 1992; Revers *et al.*, 1999).

Genome Amplification

RNA Translation

Little is known about the translation process of the potyviral RNA genome. The potyviral genome functions as mRNA, which is 3' polyadenylated, but it differs from other eukaryotic mRNA in that it lacks a 5'-cap structure, which is replaced by the VPg.

Efficient translation of most eukaryotic mRNAs requires that the 5'-cap and the 3'-poly(A) act in concert (Gallie, 1998), so an alternative mechanism must operate for potyviruses. Gallie *et al.* (1995) demonstrated that the 5' VPg interacts with

Table 1.2 Functions of potyviral gene products (Revised from Riechmann *et al.*, 1992; Revers *et al.* 1999).

Gene Product	Function(s)*
P1	*Polyprotein processing (Proteinase)* Cell-to-cell movement? Viral genome amplification?
HC-Pro	*Polyprotein processing (Proteinase)* *Vector transmission* Cell-to-cell movement *Long distance movement* *Suppressor of post transcriptional gene silencing*
P3	Polyprotein processing? (Cofactor?)
$6K_1$	Viral genome amplification?
CI	*Viral genome amplification (RNA helicase)* *Cell-to-cell movement*
$6K_2$	*Viral genome amplification*
NIa	*Polyprotein processing (Proteinase)* *RNA Replication (VPg)* Cell-to-cell movement? *Long distance movement*
NIb	*Viral genome amplification (RNA-dependent RNA polymerase?)*
CP	*Aphid transmission* *Viral RNA encapsidation* *Cell-to-cell and long distance movement*

* Functions supported by experimental evidence are in italics, those which have not are indicated with a question mark and are not in italics.

the 3'-poly(A) to promote efficient translation of *Tobacco etch virus* (TEV). Translation of eukaryotic mRNA occurs via a scanning mechanism in which initiation factors and ribosomes bind near the capped 5' end of the mRNA. The 5' cap plays a role in the binding of translation factors before the recruitment of ribosomes, so the 5' VPg must provide such a role. Wittman *et al.* (1997) have provided evidence for this by using a yeast two-hybrid system to demonstrate that the VPg of TuMV and the translation factor eIF(iso)4E from *A. thaliana* interact with each other.

It has been proposed that potyviruses should become members of the picornaviral superfamily. Members of this family lack a 5'-cap structure but have a 5'-VPg. Ribosomes have been shown to enter the RNA at an internal position via a complex secondary/tertiary structure within the 5' VPg (Pelletier & Sonnenberg, 1988). It has been suggested that potyvirus 5' VPg could act similarly (Levis & Astier-Manifacier, 1993; Basso *et al.*, 1994). Alternatively, a cap-independent "leaky scanning" mechanism has been proposed in the translation of *Plum pox virus* (PPV) genomic RNA (Riechmann *et al.*, 1991; Simón-Buela *et al.*, 1997).

Polyprotein processing

Potyviruses produce multiple proteins from a single mRNA via polyprotein processing. The NIa, P1 and HC cleave the polyprotein, see Figure 1.5 for a summary of this process (reviewed by Riechmann *et al.*, 1992). The P1 protein encodes a serine-type proteinase that catalyses the autoproteolytic cleavage between itself and HC (Verchot *et al.*, 1991). The HC encodes a cysteine-type proteinase at its C-terminus, which is responsible for the autoproteolytic cleavage between itself and the P3 protein (Carrington & Herndon, 1992). The NIa encodes a serine-type proteinase at its C-terminus, which is responsible for the remaining polyprotein processing (Carrington *et al.*, 1988).

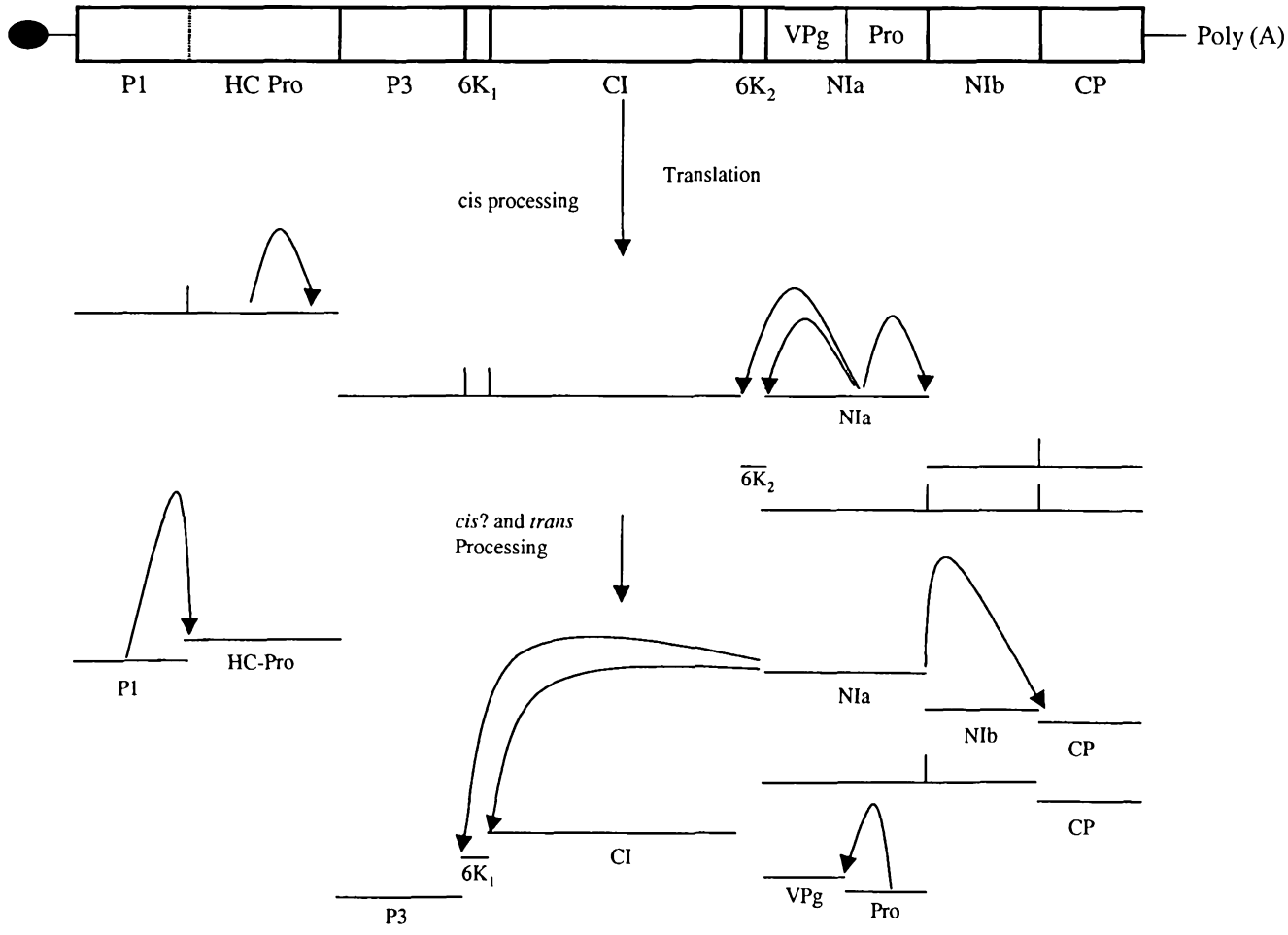


Figure 1.5 Diagrammatic representation of the potyviral polyprotein processing (Riechmann *et al.*, 1992)

RNA replication

RNA replication is initiated by the synthesis of a full-length negative strand, complementary to the genomic positive strand RNA, which is then used as a template to synthesise a positive strand RNA. Current models of replication of positive strand RNA viruses suggest that replication is catalysed by an RNA dependent RNA polymerase (RdRp) complexed to other viral and plant factors (Hayes & Buck, 1990; Duggal *et al.*, 1994).

In potyviruses, NIb, NIa and CI make up the core replicase (Riechmann *et al.*, 1992). Hong & Hunt (1996) demonstrated that the NIb protein has RdRp activity. The physical interaction between NIb and the proteinase domain of NIa in TEV (Li *et al.*, 1997; Fellers *et al.*, 1998) is important during TEV replication (Daròs *et al.*, 1999). The CI protein has RNA helicase activities (Tampo *et al.*, 1999).

The exact function of the P1 protein is not currently known but it has been shown to have some RNA binding properties (Sounounou & Laliberte, 1994), as has the NIa protein (Daros & Carrington, 1997; Merits *et al.*, 1998). It has been suggested that P1 and P3 proteins interact with the CI (Rodriguez-Cerezo *et al.*, 1993; Arbatova *et al.*, 1998). Merits *et al.* (1999) demonstrated that the P1 and P3 proteins of PVA interact with the other proteins of the replication complex. The HC also has a role in genome amplification (Kasschau *et al.*, 1997; Klein *et al.*, 1994). It has been suggested that the P1, P3 and HC proteins may be accessory factors in the genome replication process (Klein *et al.*, 1994; Verchot & Carrington, 1995; Kasschau *et al.*, 1997). The 6K proteins may also be involved in the replication process by targeting the replication complexes to membranous sites in the host (Schaad *et al.*, 1997a).

Virus Spread

To establish systemic infection, the virus moves through the plant in two distinct modes: from cell-to-cell through plasmodesmata, then long-distance through the plant via the phloem (Samuel, 1934). The achievement of systemic infection

involves the movement of the virus through several different types of cells (Figure 1.6; Carrington *et al.*, 1996). Several viral proteins are thought to be responsible for short and long-distance potyvirus movement (reviewed in Riechmann *et al.*, 1992; Carrington *et al.*, 1996; Revers *et al.*, 1999). Potyviruses are different to other plant viruses, such as *Tobacco mosaic virus* (TMV), in that they do not have a dedicated movement protein (Carrington *et al.*, 1998; Revers *et al.*, 1999).

Cell-to-cell movement

The CP, CI, VPg and HC have been implicated in potyvirus cell-to-cell movement through plasmodesmata. Evidence that the CP was required for cell-to-cell movement was provided when mutants in the CP-core domain of TEV-GUS were defective in cell-to-cell movement and virion assembly (Dolja *et al.*, 1994). Carrington *et al.* (1998) suggested that the CI was an additional intercellular movement factor of potyviruses. Evidence for both CP and CI involvement in cell-to-cell movement was also provided when both proteins were immunogold labelled and then observed at early stages of infection of tobbaco with *Tobacco vein mottling virus* (TVMV; Rodríguez-Cerero *et al.*, 1997) and *Pea seed-borne mosaic virus* (PSbMV; Roberts *et al.*, 1998). Both studies observed that that CI and CP were attached to the plasma membrane close to or over the plasmodesmal opening. It has also been suggested that the VPg protein may be involved in cell-to-cell movement (Revers *et al.*, 1999). The HC has also been implicated in the cell-to-cell movement of the potyvirus *Potato virus A* (PVA) (Andrejeva *et al.*, 1999).

Long distance movement

Viruses tend to follow a source-to-sink pattern route for translocation of photoassimilates (Andrianifahanana *et al.*, 1997). At least three viral proteins are involved in potyviral long-distance movement, CP, HC and VPg (reviewed by Revers *et al.*, 1999). Dolja *et al.* (1994), Atreya *et al.* (1995) and Andersen & Johansen (1998) demonstrated that the N and/or C terminal domains of the CP are important in the long distance movement of potyviruses. The HC has also been implicated in the long distance movement of PVA (Andrejeva *et al.*, 1999) and in

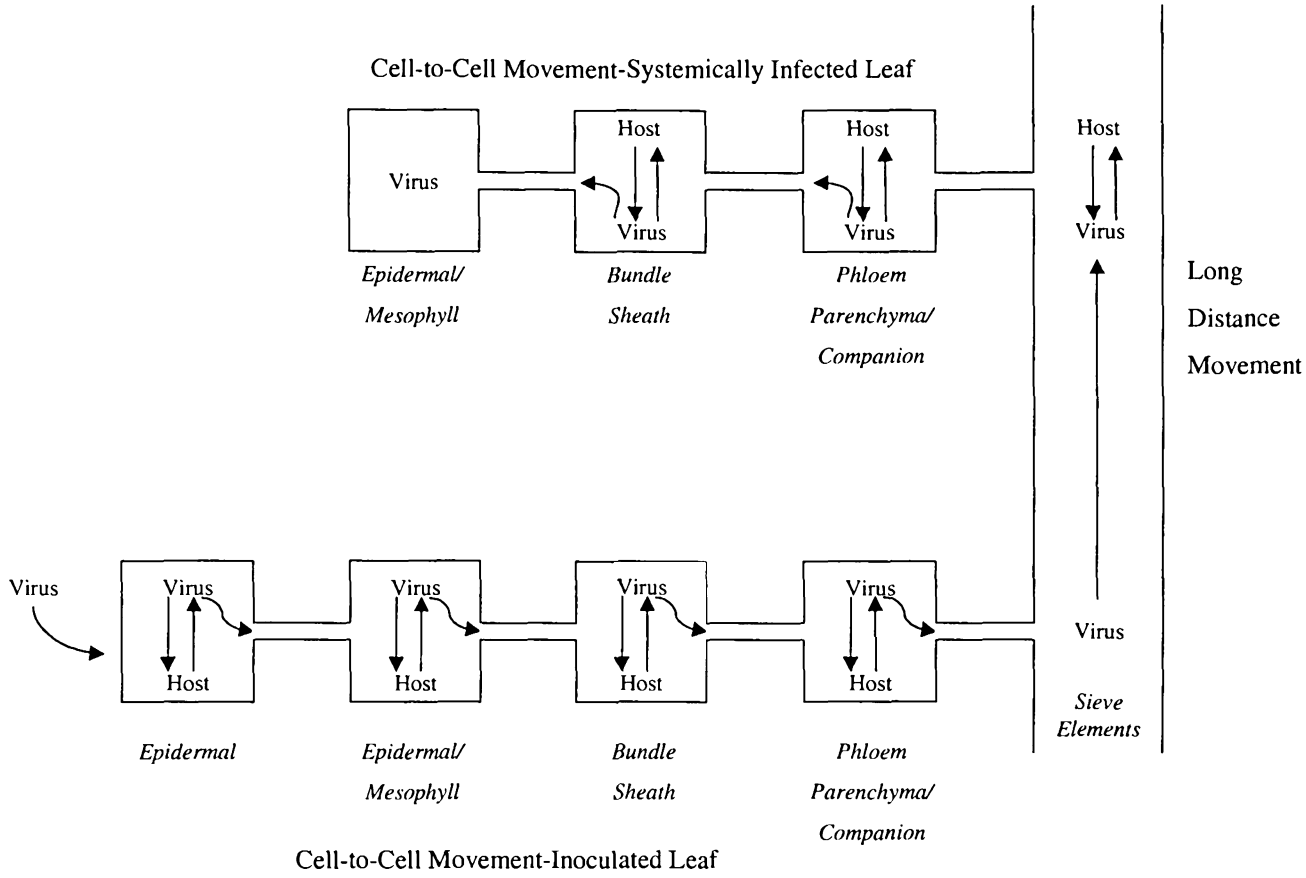


Figure 1.6 Diagrammatic representation of cell-to-cell and long distance movement pathways in plants. Interactions between virus and host and the direction of virus movement are represented by arrows. (Adapted from Carrington *et al.*, 1996).

the long distance movement of TEV (Kasschau *et al.*, 1997). The involvement of VPg and NIa in the long distance movement of TEV was proposed by Schaad *et al.* (1997b).

Potyvirus Transmission

Potyviruses are transmitted by aphids in a non-persistent manner (Brunt, 1992; Revers *et al.*, 1999). Two potyviral proteins have been shown to be involved in this process: CP and HC (Revers *et al.*, 1999). This type of transmission is dependent on the acquisition of HC prior to or together with the virus. Pirone & Blanc (1996) suggested that the HC protein acts as a bridge between virion CP and a putative receptor in the aphid mouthparts. This model has been supported by several studies (reviewed in Revers *et al.*, 1999). The main aphid vectors in the UK are thought to be *Brevicoryne brassicae* and *Myzus persicae*. A small number of potyviruses are seed transmitted. The most extensive studies have been done on PSbMV in pea (Wang & Maule, 1992, 1994; Johansen *et al.*, 1996).

Potyvirus pathogenicity determinants

The VPg protein has been identified as the virulence determinant for TEV, *Tobacco vein mottling virus* (TVMV) and PSbMV (Schaad *et al.*, 1997b). P3 has been associated with overcoming resistance to *Soybean mosaic virus* (Eggenberger & Hill, 1997) and the CP has been implicated in symptom expression (Frenkel *et al.*, 1991). Jenner *et* al. (2000) identified mutations in the TuMV-CI that resulted in virulence of normally avirulent isolates on plants possessing the mapped TuMV resistance gene *TuRB01* (Walsh *et al.*, 1999).

In summary, further investigations are required to establish a more complete understanding of all the functions of potyviral gene products and their role in the different stages of the virus lifecycle.

1.3 TURNIP MOSAIC VIRUS

Turnip mosaic virus (TuMV) is a member of the *Potyvirus* genus (Murphy *et al.*, 1995). TuMV infections of crops occur world-wide in temperate and tropical regions and the virus is transmitted in the non-persistent, stylet-borne manner by at least 89 species of aphids (Edwardson & Christie, 1986). The virus has the widest known host range of any member of the *Potyvirus* genus (Shukla *et al.*, 1994). It naturally infects arable brassica crops, edible horticultural non-brassica crops, ornamentals, members of 14 different weed families and a large number of important horticultural brassica crops - cauliflower, broccoli, cabbage, Brussels sprout, kale, kohlrabi, swede and Chinese cabbage (Shattuck, 1992). TuMV can be transmitted mechanically to an experimental host range of 318 species in 156 genera of 43 plant families (Edwardson & Christie, 1991), including *Arabidopsis thaliana* (Martin-Martin *et al.*, 1994; Martinez-Herrera *et al.*, 1994; Sanchez *et al.*, 1998). It is not known to be seed transmitted (Tomlinson, 1970).

In a survey of plant virologists TuMV was ranked the second most important virus infecting field-grown vegetables (Tomlinson, 1987). It was classed as the most important virus in North China and Taiwan and the second most important in Britain (Tomlinson, 1987). Feng *et al.* (1990) reported that 69% of *Brassica* crops sampled from Beijing were infected with TuMV. Also, Liu *et al.* (1996) found that 72% of crucifer crops were infected with TuMV and Yoon *et al.* (1993) found that 75% of Chinese cabbage samples collected from Taiwan were infected with TuMV.

TuMV induces a range of symptoms in infected plants, from chlorotic spots in leaves to systemic vein clearing, mosaic and necrosis (Shattuck, 1992). In some cases, symptoms are so severe that infection leads to plant death, especially in *B. napus* (Walsh & Tomlinson, 1985).

1.3.1 Variation of TuMV

Many TuMV isolates have been identified. Several systems for classifying TuMV isolates into groups have been developed based on the host range and symptoms induced by TuMV isolates on plant differentials (Provvidenti, 1980, 1981; Stobbs & Shattuck, 1989; Liu *et al.*, 1990; Jenner & Walsh, 1996), see Table 1.3 for summary. Serological variation (Jenner *et al.*, 1999) and sequence variation of different TuMV isolates have also been studied (Lehmann *et al.*, 1997).

Provvidenti (1980, 1981) identified TuMV strains C1-C5 based on the interaction of isolates with Chinese cabbage differentials. Green & Deng (1985), Stobbs & Shattuck (1989) and Liu *et al.* (1990) further expanded this classification system by using the Chinese cabbage differentials to identify strain C6, C7 and C8 respectively.

Another classification system, using *B. napus* rather than *B. rapa* differentials, has been developed by Jenner & Walsh (1996). One hundred and twenty four TuMV isolates from around the world, with an emphasis on Europe, were grouped into 12 groups (pathotypes) according to phenotypes induced on four *B. napus* differential lines (Table 1.4; Jenner & Walsh, 1996). Pathotypes 1, 3 and 4 were the most common (Jenner & Walsh, 1996), see Figure 1.7 for an example of the interaction of pathotype 1 isolates with the four *B. napus* differentials. The *B. napus* differential lines could not differentiate between TuMV strains C2-C5 (Provvidenti, 1980), all were classed as pathotype 3 (Jenner & Walsh, 1996). An isolate belonging to TuMV strain C1 gave the resistance/susceptibility profile of a pathotype 1 isolate (Jenner & Walsh, 1996). The inability to distinguish between C2-C5 suggests that the Chinese cabbage (*B. rapa*) differentials have different resistance genes from the *B. napus* differential lines (Jenner & Walsh, 1996). Similarly, strains Tu1-Tu5 and Tu7 (Liu *et al.*, 1990) could not be differentiated and all behaved as pathotype 3 isolates and strain Tu6 (Liu *et al.*, 1990) belonged to pathotype 1 (Jenner & Walsh, 1996).

Table 1.3 Summary of turnip mosaic virus (TuMV) classification systems.

TuMV classification	Reference
Ordinary & Cabbage	Yoshii (1963)
Type I & II	McDonald & Hiebert (1975)
C1-C4	Provvidenti (1980)
C5	Green & Deng (1985)
S1	Shattuck & Stobbs (1987)
S1-S5	Stobbs & Shattuck (1989)
C6	Stobbs & Shattuck (1989)
Pathotypes 1-4	Walsh (1989)
C7 & C8	Liu *et al.* (1990)
Tu1-7	Liu *et al.* (1996)
Pathotypes 5-12	Jenner & Walsh (1996)

Table 1.4 Twelve turnip mosaic virus (TuMV) pathotypes from TuMV interactions with four *Brassica napus* differentials. (Jenner & Walsh, 1996)

Virus pathotype	Plant line			
	S6 (oilseed rape)	R4 (oilseed rape)	165 (swede)	S1 (swede)
1	+	0	0	+
2	R	R	0	R
3	+	$+_N$	0	+
4	+	+	+	+
5	+	+	0	+
6	+	$+_N$	0	R
7	+	0	0	R
8	+	$+_N$	R_N	R
9	+	R_N	0	R
10	+	+	0	0
11	R	+	0	R
12	+	+	$+_N$	+

0, no systemic infection, plants appeared to be immune; +, systemic infection, plants were susceptible; R, local infection, no systemic infection. Symptoms were chlorotic unless indicated by $_N$ (necrotic).



Figure 1.7 Four *Brassica napus* differentials, used in the Jenner & Walsh (1996) pathotyping system, showing the typical interaction with a pathotype 1 isolate. From left to right S1 (systemic mosaic), 165 (apparent immunity), S6 (systemic mosaic) and R4 (apparent immunity).

1.3.2 Effect of TuMV on *Brassica* spp.

Within the UK, epidemics of TuMV have caused yield losses in *B. napus* crops (Tomlinson & Ward, 1978; Hardwick *et al.*, 1994). See Figure 1.8 for an example of the effect of TuMV on a *B. napus* crop. Young swede plants infected with TuMV often become stunted and unmarketable (Shattuck & Stobbs, 1987). Swede plants infected with TuMV later in development are not as severely affected as younger plants, but they are difficult to harvest and have reduced shelf life (Shattuck & Stobbs, 1987).

TuMV also causes severe yield losses in *B. oleracea* (Walkey & Webb, 1978; Walkey & Neely, 1980; Pink & Walkey, 1988). TuMV-infected cauliflowers can develop uneven curds and distorted leaves, both of which decrease marketability (Pink & Walkey, 1988). The most severely affected *B. oleracea* crop in the UK is white cabbage (Walkey & Webb, 1978; Walkey & Neely, 1980). Walkey & Webb (1978) reported that TuMV caused necrotic symptoms on the outer leaves of white cabbage and a reduction in head weight. Walkey & Neely (1980) reported that all white cabbage cultivars tested were susceptible to TuMV and that TuMV-infected cabbage heads were more susceptible to *Botrytis cinerea.* Also, development of internal necrotic symptoms, during cold storage, has been attributed to TuMV infection (Walkey & Neely, 1980; Pink & Walkey, 1990).

1.3.3 Control of TuMV

The non-persistent mode of transmission of TuMV makes it difficult to control. Inter-planting, crop rotation, crop hygiene and clean seed are effective in reducing virus infection (Walkey, 1985). In the case of TuMV, older plants appear to be less affected by TuMV infection than younger plants (Shattuck & Stobbs, 1987) so planting of crops to avoid peak aphid activity and migration would allow plants to become established before exposure to TuMV occurred (Lowery, 1988). Crops can also be planted in protected environments, e. g. glasshouses, or in areas where vectors are absent or present in low numbers.



Figure 1.8 *Brassica napus* (swede) crop infected with turnip mosaic virus (TuMV). Effect of TuMV infection was so severe that the entire crop was destroyed. Picture courtesy of J.A. Walsh, HRI Wellesbourne.

Insecticides are more effective in controlling viruses that are transmitted in a persistent rather than non-persistent manner (Walkey, 1985). Attempts to control spread of TuMV by using insecticides to control the aphid vector have been unsuccessful because the virus is transmitted in a non-persistent manner. Aphids are able to migrate and introduce TuMV into other crops before the insecticides take effect (Shattuck, 1992; Walsh, 1999).

Alternative methods of vector control include the application of reflective mulches (Lee & Lee, 1973), plant extracts (Tamura, 1969; Pandey & Mohan, 1986) or oil sprays (Bradley *et al.*, 1966; Simons, 1982). Weekly applications of mineral oil to rutabaga crops in Ontario have reduced levels of TuMV infection, but mineral oil can be phytotoxic, expensive and success is dependent upon timing and coverage (Shattuck, 1992).

The most effective control of TuMV infection may be to use host resistance in breeding programmes to breed TuMV resistant crops. To use host resistance effectively the genetic basis of resistance has to be elucidated.

1.4 HOST RESISTANCE

1.4.1 Types of Resistance

Host plant resistance to viruses can be divided into three different groups, operating at different levels within plant species (reviewed by Fraser, 1990, 1992):

Non-host resistance

Non-host resistance operates at the species level and is when all individuals in an entire species are resistant to a particular virus. No symptoms are observed and no virus can be detected upon repeated inoculations with the virus. The majority of plants are non-hosts for most viruses (Dawson & Hilf, 1992).

Acquired resistance

Acquired (or non-heritable) resistance operates at the individual level and is conferred by methods such as cross protection (where a plant is protected against more severe strains of a virus by previous inoculation with a mild strain of that virus; Salaman, 1933; Walkey, 1985; Walkey *et al.*, 1992), chemical application (Oostendorp *et al.*, 2001) or plant transformation (pathogen derived resistance, see section 1.5.5).

Cultivar resistance

Cultivar resistance operates at the individual level and has a genetic basis. Individuals within plant cultivars or lines within a species are resistant to a specific virus. This type of resistance often involves a "gene-for-gene" type interaction (see section 1.4.2) and has been most widely used in plant breeding (Crute & Pink, 1996).

1.4.2 Models of Resistance

Flor (1971) proposed the "gene-for-gene" theory to explain results of studies on the inheritance of pathogenicity in the flax rust fungus, *Melampsora lini*. The theory proposes that for resistance to occur, complementary pairs of dominant genes, one in the host and the other in the pathogen, are required. A loss or alteration to either the plant resistance (R) gene or the pathogen avirulence (Avr) gene leads to compatibility (Figure 1.9; Hammond-Kosack & Jones, 1997). Gene-for-gene type interactions have also been proposed for recessive resistance allele *sbm-1* with resistance to specific pathotypes of PSbMV (Keller *et al.*, 1998) and the recessive *bc* alleles with strain-specific resistance to BCMV (Drijfhout, 1978; Donovan, 2000).

The interaction between host plant and virus are not fully understood. Fraser (1990, 1992) proposed a theoretical model of plant-virus interaction that involves specific recognition events leading to either a susceptible or resistance response. In this model for resistance to occur two events must take place. Recognition of the virus- by host-coded molecules leads to either direct inhibition where no virus replication can occur or a recognition event that leads to a signal transduction pathway that inhibits virus multiplication and/or spread. To explain these different types of resistance responses Fraser (1990, 1992) proposed the negative and positive mechanisms:

Negative mechanism
Resistance occurs due to a lack of, or defective, plant helper factors required for virus multiplication or movement. This type of resistance is proposed to be recessive.

Positive mechanism
Resistance occurs due to an inhibitor, either already possessed by the plant (constitutive resistance) or induced after the initial recognition event (signal transduction, involving several host genes). This type of resistance is proposed to

		Host plant genotype	
		R1 r2	*r1 R2*
Pathogen genotype	*Avr1, avr2*	I	C
	avr1, Avr2	C	I

Figure 1.9 "The quadratic check." Two independently acting R-Avr gene combinations involved in R-Avr dependent incompatibility. *R1* and *R2* are two dominant plant resistance genes, where *r1* and *r2* are their respective recessive (nonfunctional) alleles. *R1* and *R2* confer recognition of pathogens carrying the corresponding pathogen avirulence genes, *Avr1* and *Avr2*, respectively, but not the situations where either the resistance gene is absent or nonfunctional (*r1*, *r2*) or the pathogen lacks or has altered the corresponding avirulence gene (*avr1*, *avr2*). I, incompatible interaction, where the plant is resistant to the pathogen; C, compatible interaction, where the plant is susceptible to pathogen attack and disease occurs (Hammond-Kosack & Jones, 1997).

be dominant or incompletely dominant. The virus should be able to become virulent by a modification in its own factor, but the viral protein must still retain its function in the virus lifecycle in order to establish systemic infection.

1.4.3 Resistance to TuMV in *Brassica* spp. and *A. thaliana*.

Resistance to TuMV in Brassica species

Resistance to TuMV, identified in *Brassica* spp., has been classified into two groups: "resistance" and "immunity" (Tomlinson & Ward, 1978, 1982; Provvidenti, 1980, 1981; Leung & Williams, 1983; Niu *et al.*, 1983; Green & Deng, 1985; Walsh & Tomlinson, 1985; Shattuck & Stobbs, 1987; Stobbs & Shattuck, 1989; Walsh, 1989; Yoon *et al.*, 1993; Anon., 1996; Jenner & Walsh, 1996; Liu *et al.*, 1996; Suh *et al.*, 1996; Anon., 1997; Walsh *et al.*, 1999; Rusholme, 2000). The term "resistance" has been used to describe the detection of virus in inoculated leaves but not in uninoculated leaves by visual assessment or other detection technique, e.g. Enzyme Linked Immunosorbent Assay (ELISA), i.e. the prevention of systemic spread of virus (Jenner & Walsh, 1996). The term "immunity" has been used to describe a more extreme form of resistance where a plant is exempt from infection (Jones & Cooper, 1984); virus is not detectable in either inoculated or uninoculated leaves. Immunity is very difficult to demonstrate, even if virus cannot be detected after repeated challenges with TuMV, by back-inoculation to indicator plants, ELISA or immunosorbent electron microscopy (ISEM) testing, virus replication could still have occurred below the detection level of assessment techniques used.

Resistance and immunity to TuMV have been identified in *B. napus* during field tests (Shattuck & Stobbs, 1987) and controlled environment tests (Tomlinson & Ward, 1978, 1982; Walsh & Tomlinson, 1985; Shattuck & Stobbs, 1987; Stobbs & Shattuck, 1989; Walsh, 1989; Walsh *et al.*, 1999). Resistance and immunity has also been identified in *B. rapa* (Provvidenti, 1980, 1981; Leung & Williams, 1983; Niu *et al.*, 1983; Green & Deng, 1985; Stobbs & Shattuck, 1989; Yoon *et*

al., 1993; Anon., 1996; Liu *et al.*, 1996; Suh *et al.*, 1996; Anon., 1997; Rusholme, 2000). Some of the TuMV resistance and immunity identified in *B. rapa* confers resistance to a wide range of TuMV isolates (Yoon *et al.*, 1993; Suh *et al.*, 1995; Anon., 1996; Anon., 1997; Rusholme, 2000). TuMV resistance and immunity identified in *B. napus* appears to be strain-specific (Tomlinson & Ward, 1978, 1982; Walsh & Tomlinson, 1985; Shattuck & Stobbs, 1987; Stobbs & Shattuck, 1989; Walsh, 1989; Walsh *et al.*, 1999).

In contrast to *B. napus* and *B. rapa*, little TuMV resistance has been reported in *B. oleracea*. Partial forms of resistance have been observed in some cultivars of white cabbage (Walkey & Pink, 1988). Pink *et al.* (1986) observed mild systemic symptoms in inbred lines of Brussels sprouts and Pink & Walkey (1988) were unable to identify any immune forms of cauliflower. Mild systemic symptoms might indicate that a plant is TuMV tolerant, allowing the virus to replicate and spread without causing severe symptoms or significantly reducing plant growth or yield (Jones & Cooper, 1984). The marketability of some crops, such as cabbage and cauliflower, can be severely affected by the appearance of mild symptoms. Tolerant cultivars can also act as reservoirs of virus inoculum.

Resistance to TuMV in Arabidopsis thaliana

As previously mentioned, TuMV infects *A. thaliana*. Martin-Martin *et al.* (1999) inoculated 106 *A. thaliana* ecotypes with TuMV isolate UK 1 and identified four ecotypes that were resistant. The resistance in one of the ecotypes, Bay-0, appeared to interfere with cell-to-cell movement. Resistance to another potyvirus, TEV, has also been identified in *A. thaliana* (Mahajan *et al.*, 1998). Two genes, *RTM1* and *RTM2*, necessary for resistance to TEV have been cloned (Chisholm *et al.*, 2000; Whitham *et al.*, 2000).

1.4.4 Genetic basis of resistance to TuMV in *Brassica* spp.

Strain-specific TuMV resistance in *B. napus* appears to be controlled by single dominant genes (Shattuck & Stobbs, 1987; Bradshaw *et al.*, 1989; Walsh, 1989; Walsh *et al.*, 1999). One of these TuMV resistance genes, *TuRB01* (*TuMV resistance in Brassica 01*), has been mapped to the A genome linkage group N6 on a *B. napus* genetic linkage map and is the first gene for resistance to TuMV to be mapped in a *Brassica* species. Walsh *et* al. (1999) also mapped a QTL for TuMV resistance, *TuRB02* (*TuMV resistance in Brassica 02*), to C genome linkage group N14. Prior to the identification and mapping of *TuRB01* and *TuRB02*, the only gene controlling resistance to TuMV that had been mapped was the *Tu* gene in *Lactuca sativa* (Montesclaros *et al.*, 1994).

Studies on the inheritance of TuMV resistance in Chinese cabbage reported that TuMV resistance was controlled by dominant resistance genes (Leung & Williams, 1983; Niu *et al.*, 1983), but the assessments of infection did not include inoculation to an indicator plant or ELISA testing to confirm visual assessments. More recent experiments on the genetic basis of broad-spectrum TuMV resistance in Chinese cabbage have included ELISA tests to confirm visual assessments (Yoon *et al.*, 1993; Suh *et al.*, 1995; Anon., 1996, 1997; Rusholme, 2000) and back inoculation to susceptible indicator plants (Rusholme, 2000). Rusholme (2000) also inoculated four *B. napus* differentials (Jenner & Walsh, 1996) to confirm that TuMV isolates were behaving as expected.

Yoon *et al.* (1993) and Suh *et al.* (1995) used the same resistant Chinese cabbage parent in crosses to different susceptible parents. Yoon *et al.* (1993) concluded that two recessive genes were required for resistance to TuMV strains C4 and C5 but it was not clear if the same genes were required for resistance to both strains. Suh *et al.* (1995) identified dominant genes controlling resistance to TuMV strains C4 and C5, the number of dominant genes controlling resistance was either one or two depending on the susceptible parent used in crosses. Segregation ratios produced from virus testing one cross combination could not be explained by

Mendelian genetics. The different conclusions of Yoon *et al.* (1993) and Suh *et al.* (1995) could be due to using different susceptible parents with different genetic backgrounds. One of the susceptible parents used by Suh *et al.* (1995) possessed resistance to strain C1. In addition, although both studies used the TuMV strain identification system of Provvidenti (1980), neither paper mentions using the differential lines of Provvidenti (1980) to verify the strain classification of the TuMV isolate used. Different methods of inoculation, detection of virus or environmental conditions during the experiments could also affect results.

Suh *et al.* (1996) found that segregation ratios produced from inoculating Chinese cabbage with a mixture of TuMV strains could not be explained by Mendelian genetics and suggested that this could be due to the possible existence of minor modifying genes involved in resistance to multiple strains of TuMV. Inoculation of mixed virus isolates can result in interactions between the isolates, for example, infection with one virus strain can prevent infection by different strains of the same or a similar virus, this is successfully exploited in cross-protection of crops (Salaman, 1933). Another example is when one virus may facilitate the successful infection of an avirulent virus, e.g. *Pepper mottle potyvirus* successfully infects normally resistant *Capsicum annum* cv. Avelar plants upon co-infection with *Cucumber mosaic cucumovirus* (Guerini & Murphy, 1999).

Rusholme (2000) characterised two types of TuMV resistance in Chinese cabbage: strain specific immunity and broad-spectrum resistance. The dominant allele, *TuRB01b*, which conferred strain specific immunity, had a similar specificity of interaction and mapped to the same position on A-genome linkage group N6 as the previously mentioned *TuRB01*. The broad-spectrum resistance appeared to be controlled by a combination of one recessive and one dominant allele, which mapped to A-genome linkage groups N4 and N8 respectively.

As previously mentioned, little resistance to TuMV has been identified in *B. oleracea*. Attempts to genetically characterise the partial resistance or tolerances identified in *B. oleracea* suggest that partial or incompletely dominant genes were

responsible for resistance (Tomlinson & Ward, 1981; Pink *et al.*, 1986). However, none of the TuMV isolates used were identified as certain strains or pathotypes of TuMV and it is therefore difficult to compare the results generated. Tomlinson & Ward (1981) confirmed visual assessments with inoculation to an indicator plant and ISEM.

From the studies above it appears that simply inherited genes conferring strain-specific TuMV resistance occur in both *B. rapa* and *B. napus*. Broad-spectrum resistance to TuMV isolates appears to occur in *B. rapa* and the control of these resistances appears to be under complex genetic control, although the genetic basis is not fully understood and requires further investigation. The tolerance of TuMV in *B. oleracea* also appears to be under more complex, multigenic control. The lack of simply inherited TuMV resistance genes in *B. oleracea* could indicate that the strain-specific, simply inherited resistance genes are located predominantly on the A-genome of *B. rapa* and *B. napus*. Single dominant alleles conferring strain-specific TuMV resistance and monogenic dominant and recessive alleles conferring broad-spectrum TuMV resistance mapped to the A-genome (N6, N4 and N8 respectively) (Walsh *et al.*, 1999; Rusholme, 2000). Conversely, the QTL, *TuRB02*, mapped to the C-genome (N14) of *B. napus* (Walsh *et al.*, 1999).

1.5 PLANT MOLECULAR BIOLOGY

Advances in plant molecular biology over the past decade have increased knowledge about the structure, function and mechanism of action of plant disease resistance genes. The DNA-based marker techniques have several advantages over traditionally used morphological markers and are constantly being modified to enhance their utility. The development of the polymerase chain reaction (PCR) technique has brought about a new class of DNA profiling markers (reviewed by Joshi *et al.*, 1999). DNA-based markers can be divided into two groups: single (or low copy) probes or multi locus probes

1.5.1 Single locus probes

Restriction fragment length polymorphisms (RFLP)

RFLP are simply inherited naturally occurring Mendelian characters. They are produced when restriction enzymes cleave genomic DNA, generating smaller fragments, which are resolved by agarose gel electrophoresis, then are blotted on to a nitrocellulose membrane. The fragments are then visualised by hybridisation of radioactively labelled DNA probes to homologous restriction fragments. The length of restriction fragments can vary between individuals due to changes in the sequence of the restriction site, the formation of a new restriction site, or insertions or deletions within the fragment generated. The advantages of RFLP markers are that they are codominant, phenotypically neutral, reliable in linkage analysis, often detect high levels of allelic variation and are free from environmental and epistatic effects.

Several RFLP maps have been generated for: *B. napus* (Landry *et al.*, 1991; Ferreira *et al.*, 1994; Uzunova *et al.*, 1995; Parkin *et al.*, 1995; Sharpe *et al.*, 1995), *B. rapa* (Song *et al.*, 1991; Chyi *et al.*, 1992; Kole *et al.*, 1997; Rusholme

2000), *B. oleracea* (Slocum *et al.*, 1990; Landry *et al.*, 1992; Bohoun *et al.*, 1996), *B. nigra* (Lagercrantz & Lydiate, 1996) and *B. juncea* (Cheung *et al.*, 1997).

1.5.2 Multi locus probes

Random amplified polymorphic DNA

The RAPD technique is based on PCR. RAPD markers are produced using a primer of arbitrary nucleotide sequence to amplify unrelated loci from across the genome (Welsh & McClelland, 1991). The PCR products are resolved by agarose gel electrophoresis and visualised by ethidium bromide staining. Many loci can be generated rapidly in this way with polymorphisms resulting from sequence differences in one or more of the primer annealing sites. RAPD markers are dominant markers, so have limitations in their use as markers for mapping, and are sensitive to environmental variation so are not highly reproducible between laboratories (Penner *et al.*, 1993).

Amplified fragment length polymorphism (AFLP)

The AFLP technique (Vos *et al.*, 1995) involves the detection of genomic restriction fragments by PCR amplification. The genomic DNA is cleaved with two restriction enzymes, usually *Eco*RI and *Mse*I then oligonucleotide adapters are ligated to the restriction fragments. The adapters consist of a core sequence and an enzyme specific sequence. Selective primers that consist of three parts: a core sequence, an enzyme sequence (complementary to either *Eco*RI or *Mse*I) and a selective extension are used to amplify a subset of the fragments. The primers with enzyme sequences complementary to *Eco*RI are either radioactively or fluorescently labelled. The PCR products are resolved using high-resolution gel electrophoresis and are visualised using either autoradiographs or an automated fluorescence based detection system (Hartl & Seefelder, 1998; Zhang *et al.*, 2000). An additional selective amplification step is included when complex plant

or animal DNA fingerprinting is required. The primers can have different selective nucleotide extensions to generate new AFLP patterns.

The AFLP technique is similar to the RAPD technique in that it requires small quantities of DNA and it produces dominant markers, yet the stringent reaction conditions applied in the AFLP technique makes it more reproducible than RAPDs. The AFLP technique is also similar to the RFLP technique in that it resolves restricted DNA fragments. However, it has several advantages over RFLP because it is less time consuming, requires less DNA and, if the fluorescent technique is used, it is less hazardous and less expensive. AFLPs have a further advantage over RFLP in that they have the potential to detect large numbers of polymorphic loci. AFLPs have been successfully used in bulked segregant analysis (Michelmore *et al.*, 1991; Thomas *et al.*, 1995).

Simple sequence repeats (microsatellites)

Simple sequence repeats (microsatellites) are tandem repeats with a monomer sequence of between one and six base pairs which is repeated several times (Joshi *et al.*, 1999). Polymorphism is produced when the numbers of repeated units vary between genotypes. The polymorphism is detected by PCR amplification of genomic DNA using two unique primers adjacent to the tandem repeat unit. The amplified products are resolved by gel electrophoresis. The products have traditionally been visualised using radioactive labelling but the development of automated, fluorescence based detection systems has made it safer. Fluorescent systems also allow several primer pairs to be combined in a single amplification reaction (multiplexing), which reduces the time in which a large volume of results can be generated. Microsatellite markers are codominant, detect multiple alleles and are highly reproducible. They are becoming more widely used and have been identified and mapped in several different species, for example *Arabidopsis* (Bell & Ecker, 1994), wheat (Roder *et al.*, 1998), *B. oleracea* (Sebastian *et al.*, 2000), *B. rapa* and *B. napus* (A. Sharpe & D. Lydiate, personal communication).

1.5.3 Plant Disease Resistance Genes

The development of plant molecular biology techniques has recently led to the isolation of several R genes from different species conferring resistance to a variety of plant pathogens (Table 1.5). The isolation, or cloning, strategies relied predominantly upon defining the chromosomal location of the genes, e.g. by molecular mapping, then identifying the correct sequence by either transposon insertion to destroy biological activity or cosmid complementation to restore the resistance phenotype (Hammond-Kosack & Jones, 1997). From these sequences, predictions about R protein structure and function can be made.

The isolated R genes confer resistance to a number of different types of pathogens, but the majority of the predicted protein sequences have one or more structural motifs in common. The R genes can be divided into groups depending on the predicted structure of the proteins they encode (reviewed by Bent, 1996; Hammond-Kosack & Jones, 1997; Holub, 2001; Staskawicz, 2001).

NADPH-dependent reductase

The *Hm1* gene from maize was the first disease resistance gene to be cloned and characterised (Bent, 1996; Hammond-Kosack & Kones, 1997; Staskawicz, 2001). Johal & Briggs (1992) isolated *Hm1* by transposon tagging, subsequent characterisation revealed that it encoded an NADPH-dependent reductase that inactivates the fungus *Cochliobolus carbonum.*

Serine Threonine Kinase

Martin *et al.* (1993) used a map-based cloning strategy to isolate the *Pto* resistance gene from tomato. Subsequent characterisation of the *Pto* gene revealed that the encoded protein was predicted to have serine/threonine kinase activity. The *Pto* gene was completely unrelated to the *Hm1* gene.

Table 1.5 Cloned plant disease resistance genes (adapted from Hammond-Kosack & Jones, 1997). NBS, nucleotide binding site; LRR, leucine rich repeat motif; LZ, leucine zipper motif; TIR, toll/interleukin-1 homology;

Gene	Plant	Pathogen	Predicted Features of R protein
Hm1	Maize	*Helminthosporium maydis* (race 1)	NADPH-dependent reductase
Pto	Tomato	*Pseudomonas syrinage* p.v. tomato (avrPto)	Serine Threonine Kinase
RPS2	Arabidopsis	*Pseudomonas syringae* p.v. tomato (avrRpt2)	NBS-LRR-LZ
RPM1	Arabidopsis	*Pseudomonas syrinage* p.v. maculicola (avrRpm1/avrB)	
I_2	Tomato	*Fusarium oxysporum* f.sp. *lycopersicon*	
Prf	Tomato	*Pseudomonas syringae* pv. tomato (avrPto)	
N	Tobacco	TMV	NBS-LRR-TIR
L6, M	Flax	*Melampsora lini (AL6, AM)*	
RPP5	Arabidopsis	*Peronospora parasitica*	
Rx	Potato	PVX	
Cf-9, Cf-2, Cf-4, Cf-5	Tomato	*Cladosporium fumvum (Avr9, Avr2, Avr4, Avr5)*	LRR
Xa-21	Rice	*Xanthomonas oryzae* p.v. *oryzae* (all races)	Transmembrane Receptor Kinases

The Leucine Rich Repeat Classes

The largest class of plant disease resistance proteins is the leucine rich repeat class. Since 1994, several genes have been cloned that confer resistance to a diverse array of plant pathogens, each with a predicted encoded protein structure including a LRR motif. LRRs are multiple serial repeats of a motif approximately. 24 amino acids in length and have been predicted to be involved in protein-protein interactions in animals (Kobe & Deisenhofer, 1994) and it is hypothesised that they may be responsible for recognition specificity in gene-for-gene interactions (Bent, 1996; Jones & Jones, 1997). The LRR class can be divided into two groups: those that encode a nucleotide binding site (NBS) and those do not encode a NBS (non-NBS).

Nucleotide Binding Site

NBS domains occur in proteins with ATP or GTP binding activity (Bent, 1996). The presence of highly conserved NBS domains in some *R* gene products suggests that they are essential for the functioning of these proteins.

The NBS-LRR class of genes can be further subdivided into two subgroups, those with toll/interleukin-1 receptor homology (TIR class) and those without (non-TIR class).

The *N*, *L6*, *M* and *RPP5* genes encode a large N-terminal domain with similarity to the cytoplasmic signalling domain of the Drosophila Toll protein and mammalian interleukin-1 receptors (Whitham *et al.*, 1994; Lawrence *et al.*, 1995; Hammond-Kosack & Jones, 1997; Parker *et al.*, 1997). The similarity of the cytoplasmic signalling domains of these receptors and the N, L6, M and RPP5 proteins implies that these plant disease resistance proteins are involved with signalling and not ligand binding (Hammond-Kosack & Jones, 1997).

The *RPS2*, *RPM1*, *Prf*, I_2 and *Rx* predicted gene products have a possible leucine zipper region (LZ) (Bent, 1996; Hammond-Kosack & Jones, 1997). It is proposed

that LZ domains facilitate the formation of a coiled-coil structure to promote either dimerization or specific interactions with other proteins.

Non-NBS

The proteins encoded by *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* do not contain an apparent NBS. The genes encode LRRs and analysis of the derived amino acid sequence suggests that the LRRs are normally extracellular. The N terminus of Cf-9 includes a probable signal peptide sequence for transport across membranes; the C-terminus consists of an apparent transmembrane domain and a short amino acid tail that is probably cytoplasmic.

Transmembrane Receptor Kinases

Xa21 encodes an apparent LRR receptor kinase in which the N-terminal LRRs are likely to be arranged extracellularly. The external LRR domain is thought to be joined by a transmembrane region to a cytoplasmically oriented protein kinase domain.

In summary, the structural similarities between many cloned R genes suggest they may share common mechanisms against a wide variety of pathogens (Staskawicz, 2001). The transformation of tomato with the *N* gene from tobacco provided effective resistance against TMV and indicated that the common structure of many R genes is likely to facilitate the successful transfer of genes across species barriers (Erickson *et al.*, 1999a).

1.5.4 Defence Responses

The first stage in plant disease resistance is to recognise the pathogen elicitors. The LRR group is most likely to be the "pathogen recognition" class of resistance genes, because the LRR structural motif has been implicated in protein-protein interactions (Kobe & Deisenhofer, 1994). Erickson *et al.* (1999b) provided

evidence for this when they demonstrated that the *N* product interacted with the TMV replicase product in the cytoplasm.

Once recognition occurs a resistance signalling pathway is initiated. The LRR domain may also participate in signalling transduction cascades (Baker *et al.*, 1997). The hypersensitive response (HR) is the most common local resistance response in gene-for-gene type interactions, but it is not the only response accompanying incompatible interactions. The classic response in HR is localised cell death at the site of infection, resulting in the formation of a necrotic lesion, which prevents spread of the pathogen (Dangl *et al.*, 1996). Resistance to TMV conferred by the *N* gene in tobacco is probably the most characterised gene-for-gene system that induces an HR (Erickson *et al.*, 1999a). The local resistance response in potato, conferred by *Rx*, has been shown to be an extreme form of resistance that is epistatic to a hypersensitive response (Bendahmane *et al.*, 1999).

The formation of a lesion, either through HR or as a symptom of disease, induces a signal transduction pathway, systemic acquired resistance (SAR) (Ryals *et al.*, 1996). SAR plays an important role in the ability of plants to defend themselves against pathogens. It is long lasting and often enables the plant to become resistant to other plant pathogens. Salicylic acid (SA) plays a key role in SAR and disease resistance (Ryals *et al.*, 1996; Murphy *et al.*, 2001). The NBS/LRR and serine/threonine kinases are most likely to be involved in signal transduction pathways (Hammond-Kosack & Jones, 1997),

1.5.5 Application of Plant Molecular Biology to control TuMV

Conventional Breeding

Plant breeders have successfully used conventional plant breeding strategies to introgress disease resistance, often conferred by single genes, into crop varieties. However, the rapid evolution of matching pathotypes on previously resistant cultivars has forced breeders to continually replace cultivars with new resistance specificities (boom and bust cycle; Crute & Pink, 1996; Pink & Puddephat, 1999).

The pyramiding of resistance genes can lessen the chance of viruses overcoming resistance. Molecular markers could be deployed via marker assisted selection (MAS) for rapid introgression of one or more desirable disease resistance genes to form pyramids of genes for durable disease resistance (Young, 1999).

Engineered Resistance

Conventional breeding strategies can involve lengthy backcross breeding programmes to eliminate deleterious genes (Pink & Puddephat, 1999). The deployment of disease resistance genes may be achieved through plant transformation and disease resistance genes introduced directly into elite germplasm without the introduction of deleterious genes. As for conventional breeding programmes, effective deployment of disease resistance genes in transgenic crops to avoid the "boom-bust" cycle would have to be considered.

Engineering resistance to viral pathogens has been suggested as an alternative to the use of plant disease resistance genes. Some viral derived genes have already been inserted into susceptible plants to provide resistance (pathogen-derived resistance; reviewed by Wilson, 1993). The commercial deployment of transgenic virus-derived resistance has involved coat-protein mediated resistance (Fuchs & Gonsalves, 1995; Birch, 1997) where over expression of the viral coat-protein gene, incorporated in the plant genome, prevents establishment of viral infection. Several possible hazards have been suggested for coat-protein mediated resistance: transencapsidation, recombination and synergism (Robinson, 1996), all of which may result in novel viruses being produced. Transencapsidation is when an infecting viral genome is encapsidated by transgenically expressed coat protein and has been observed for TMV (Holt & Beachy, 1991) and PVY (Farinelli *et al.*, 1992).

1.6 AIMS AND OBJECTIVES

There were four main aims of this project:

The first aim was to develop experimental techniques with the long-term goal of screening *A. thaliana* ecotypes for resistance to TuMV.

The second aim was to identify TuMV resistance in *B. rapa* and *B. napus*. Jenner & Walsh (1996) classified 124 TuMV isolates from around the world into 12 pathotypes using four *B. napus* differentials. Of these pathotypes 1, 3 and 4 were the most prevalent. The first objective within this aim was to identify *Brassica* lines that were resistant to isolates representing these three prevalent pathotypes. The pathotyping system could not distinguish strains C2-C5, all were classified as pathotyope 3 (Jenner & Walsh, 1996), whereas they could be differentiated by another differential system (Provvidenti, 1980). The second objective was to identify *Brassica* lines that could differentiate between an isolate representing strain C5 and the representative of pathotype 3. The third objective was to identify *Brassica* lines with unique resistance specificities of interaction with any of the four TuMV isolates used.

The third aim was to characterise the genetic basis of TuMV resistance in lines that were either resistant to all isolates used, could differentiate between C5 and the representative of pathotype 3 or had novel resistance specificities of interactions.

The fourth aim was to identify molecular markers linked to TuMV resistance gene(s). The molecular markers were then used to position the genes on *B. napus* genetic linkage maps.

CHAPTER 2

TURNIP MOSAIC VIRUS - *ARABIDOPSIS THALIANA* INTERACTIONS

2.1 INTRODUCTION

Arabidopsis thaliana, a member of the family *Brassicaceae*, is a small annual with typical characteristics of an annual weed, minimum phenotype required for survival and a straightforward lifecycle. It has been used for model systems since the 1950's (Pigliucci, 1998) due to several advantages over other plants. It is easy to grow at high density due to its small size, while its rapid lifecycle makes it ideal for phenotypic studies. Its small genome size, small number of chromosomes and ease of transformation make it ideal for molecular analysis. *A. thaliana* is often associated with the economically important *Brassica* genus due to islands of conserved organisation between both genomes (Kowalski *et al.*, 1994; Lagercrantz *et al.*, 1996; Cavell *et al.*,1998; Conner *et al.*, 1998) despite the duplicated and rearranged nature of the *Brassica* genome relative to that of *A. thaliana* (Kowalski *et al.*, 1994).

TuMV has been shown to infect *A. thaliana* (Martinez-Herrera *et al.*, 1994) and sources of resistance to TuMV have been identified (Martin-Martin *et al.*, 1999). Resistance sources have already been identified to another potyvirus, tobacco etch virus (TEV), in *A. thaliana* (Mahajan *et al.*, 1998).

It would be advantageous to screen *A. thaliana* for resistance because gene(s) conferring resistance in *A. thaliana* would be easier to characterise than those found in *Brassica* spp. (Walsh *et al.*, 1999; Rusholme, 2000) due to the small size of *A. thaliana*, it's rapid generation time and the many molecular tools available. It would be beneficial to assess the interaction of TuMV isolates with plant lines that had been used as parents of populations developed for genetic maps. These

genetic maps and seed of subsequent generations (F_1, F_2 etc.) could be used for characterisation of any TuMV resistance identified.

This chapter describes the optimisation of experimental conditions - temperature, mechanical inoculation technique and assessment of TuMV infection. Finally it describes the screening of several *A. thaliana* ecotypes for resistance to several TuMV isolates using the optimised conditions.

2.2 OPTIMISING PROCEDURES FOR INFECTING *A. THALIANA* AND ASSESSING INTERACTIONS WITH TURNIP MOSAIC VIRUS

2.2.1 Introduction

Martinez-Herrera *et al.* (1994) and Martin-Martin *et al.* (1999) used similar environmental conditions to assess TuMV interaction with *A. thaliana.* The environmental conditions available at HRI to conduct experiments on *A. thaliana* are different to those used by Martinez-Herrera *et al.* (1994) and Martin-Martin *et al.* (1999). In the following experiment the available temperatures (18°C and 22°C) will be compared to identify the optimal temperature for assessment of TuMV infection by visual assessment and ELISA.

2.2.2 Materials & Methods

Plant material

Differential lines developed for screening *Xanthomonas* spp. and *Perenospora* spp. (E. Holub, personal communication) were used in this experiment and are listed in Table 2.1. These lines were chosen because they include parents of mapping populations and mutants that have shown different interactions with other pathogens when compared with wild-types.

Virus isolate

The type member of pathotype 1, UK 1, was used in this experiment. This isolate was selected because it has been used to screen extensive collections of *Brassica oleracea, B. rapa*, and *B. napus* accessions and was used by Martinez-Herrera *et al.* (1994) and Martin-Martin *et al.* (1999) to screen *A. thaliana* accessions. It is indigenous to the UK. The virus isolate was maintained in a susceptible host *B.*

Table 2.1 Results from testing *A. thaliana* differential lines with turnip mosaic virus isolate UK 1 at 18°C.

Plant Line	Symptoms[1]	Mean A_{405} from ELISA[2]
Oy-1	pv ld sc	2.29
Ws-*eds 1*	pv ld sc	2.77
Ws-*rpp10*	pv sc	2.73
Nd-*rpp1* 8502	pv sc	2.81
Wei-1	pv sc	2.43
Ler-0	sc	2.32
Col-5	sc	1.95
Nd-*rpp13* 8573	sc	2.81
Ksk-1	sc	2.37
Nd-1	pv sc	2.29
Ws-3	pv sc	2.15
Nd-*eds* 8777	sc	2.90
Col-γ 1-2	pv sc	2.34
Col-*pad4*	pv sc	2.57
Col-*ndr1*	pv sc	2.87
Col-*rps5* ems2	pv sc	2.73

Notes: [1] pv: purple veins and mottling
ld: leaf distortion
sc: systemic chlorosis
[2] mean of readings from two ELISA plate wells

juncea cultivar (cv.) Tendergreen (TGM) by mechanical inoculation (Jenner & Walsh, 1996), in an insect-proof glasshouse at 18°C.

Plant cultivation

Seeds of each differential line were sown in a compost mixture (6 parts Levington F2 compost : 1 part sand : 1 part vermiculite, all sieved with a 5mm sieve). Seed was planted in seed trays and left for two weeks at 18°C, 10-hour day length. Plants were then transferred into individual P40 modules and grown at 18°C, 10-hour day length.

Inoculation procedure

At four weeks old, plants were dusted with carborundum and three leaves were marked by using a glass pasteur pipette to punch a hole in them. Virus inoculum was prepared by grinding 1g systemically infected TGM leaves in 2ml solutions of K_2HPO_4 ($10g.l^{-1}$) containing Na_2SO_3 ($1g.l^{-1}$). The three marked leaves were mechanically inoculated using a cotton bud, saturated with sap diluted in inoculation buffer, as the inoculation instrument. Five plants were inoculated per differential line. Control plants were inoculated with K_2HPO_4 ($10g.l^{-1}$) containing Na_2SO_3 ($1g.l^{-1}$). Batches of test plants were maintained at 18°C or 22°C, 10-hour day length for the duration of the experiment.

Assessment of infection

Symptoms were assessed weekly by visual inspection and recorded for up to three weeks post-inoculation. After three weeks, whole plants were ground and the sap tested for the presence of TuMV using a plate-trapped antigen (PTA) ELISA test, similar to that described by Jenner & Walsh (1996). The sap was diluted 1:10 in coating buffer (Na_2CO_3, $1.6g.l^{-1}$, $NaHCO_3$, $30g.l^{-1}$). 100µl of the diluted sap was loaded into duplicate wells on a microtitre plate. After an overnight incubation at

4°C the plates were washed 3 times for 3 minutes in phosphate buffered saline ($Na_2HPO_4.12H_2O$, 2.9g.l^{-1}; KH_2PO_4, 0.2g.l^{-1}; NaCl, 8g.l^{-1}; KCl, 0.2g.l^{-1}) containing 0.5% Tween-20 (PBS-T, pH7.3). The plates were then coated with TuMV monoclonal antibody EMA67 (Jenner *et al.*, 1999), diluted 1/2500 in PBS-T containing 0.05% B-SA and incubated at room temperature for 2 hours. After incubation, the plates were washed in PBS-T (as described above) and coated with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma A-3562), diluted 1/5000 in PBS-T containing 0.05% B-SA. The plates were incubated for a further 2 hours at room temperature. After incubation the plates were washed (as described above) and the colour reaction developed by adding 100µl substrate buffer (diethanolamine, 97ml.l^{-1} pH9.8) to each well. The reactions were read with a Titertek Multiskan MCC/340 plate reader at an absorbance of 405nm. In all ELISA tests the healthy controls were used as blanks and a reaction was considered positive if the absorption value was above 0.300.

2.2.3 Results

Symptoms appeared very rapidly and the majority of inoculated plants of all differential lines were dead at the end of three weeks when maintained at 22°C. Plants that had survived were symptomless and virus was undetected in ELISA. Plants kept at 18°C showed symptoms and were ELISA positive at the end of three weeks (Table 2.1). All lines were susceptible to UK 1 (Table 2.1). No necrosis was observed; all infected plants had chlorotic symptoms.

2.2.4 Discussion

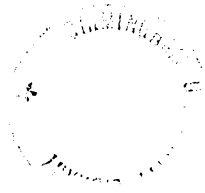
A range of differential *A. thaliana* lines were used to increase the possibility of different interactions occurring with the TuMV isolate UK 1, thus maximising potential for information to be gained about which temperature was optimal for assessment of infection. The results show that a range of plant-virus interactions occurred. The results in Table 2.1 show that slightly different symptoms were

observed and that the A_{405} values from ELISA were similar to each other. All lines used in this experiment were susceptible to TuMV isolate UK 1.

The results from this experiment show that 18°C was more suitable for studying TuMV-*A. thaliana* interactions than 22°C. Symptoms appeared too quickly for phenotypic assessments to be made accurately at the higher temperature. At the end of the assessment period the majority of plants were dead when maintained at 22°C so it was not possible to carry out ELISA tests on them. One of the reasons for screening *A. thaliana* with TuMV is to use it as a model system to gain more information about movement of the virus in plants. It is important to conduct experiments at a temperature that will allow assessment of symptom development over a long period of time. Plants kept at 18°C provided the opportunity to study symptom expression over a longer period of time so will be used as the experimental temperature in the future. This is also coincident with the temperature used for *Brassica* screening experiments.

The use of cotton buds has proved to be an ineffective tool for mechanical inoculation of *A. thaliana* with TuMV. Although all plants maintained at 18°C were infected, some plants maintained at 22°C were not infected. It is important for all plants to be infected at both temperatures to ensure that the inoculation technique is reliable. All the *A. thaliana* differentials are inbred lines (E. Holub, personal communication), so plants of the same line were expected to interact with the virus in the same way. This made escapes easy to identify. Escapes are more difficult to identify when screening wild ecotypes because they are more heterogeneous than the inbred lines developed in laboratories. It is possible that wild ecotypes are under selection pressure from TuMV infection. Consequently, it may be that resistance is more prevalent in such ecotypes than in lab accessions. It is important to have an inoculation technique that produces 100% infection of susceptible plants so that wild ecotypes can be screened with confidence. The small size of *A. thaliana* plants makes them difficult to handle so the inoculation technique also has to be simple.

The *A. thaliana* differential lines tested in this experiment were all susceptible to UK 1, but they showed different phenotypes upon infection (Table 2.1). Systemic chlorosis was the most common symptom. The lack of necrosis in these lines was particularly noticeable because this is readily observed in the interaction between some *Brassica* spp. and some TuMV isolates.



2.3 IMPROVEMENT OF THE INOCULATION PROCEDURE AND METHOD OF ASSESSMENT OF TUMV INFECTION

2.3.1 Introduction

As the inoculation procedure using cotton buds did not provide 100% infection of susceptible plants (section 2.2), it was considered important to improve the inoculation technique to produce 100% infection. It was also considered important to assess infection by consistent methods, visually and by ELISA. TuMV resistance genes identified in *A. thaliana* could be compared to resistance genes found in *Brassica* (Chapter 3). Similar assessment of infection could aid the comparison of results between the two species. Three phenotypic classes have been described in the *Brassica* system: 0, R, + (Jenner & Walsh, 1996). It would therefore be a good idea to develop a method of assessment that would allow these phenotypes to be identified in *A. thaliana*. The R phenotype, resistance to systemic spread of virus, has been identified in *A. thaliana* against TEV (Mahajan *et al.*,1998), so it is possible this type of resistance exists against TuMV.

2.3.2 Materials & Methods

Plant Material

Seed for ecotypes col-γ 1-2 and col-*ndr1*, used in this study, were obtained from E. Holub, HRI, Wellesbourne, UK.

Virus isolate

UK 1 was used in this experiment because it is indigenous and it is known to be virulent from the results of the experiment described in section 2.2 and other research (Martinez-Herrera *et al.*, 1994; Martin-Martin *et al.*, 1999).

Inoculation technique

Plants were grown as described in section 2.2. Once they had been transferred to P40 modules the plants were placed in a random block design (Figure 2.1). At four weeks old three leaves were marked and inoculum prepared as described in section 2.2. Marked leaves were inoculated by lightly rubbing them with muslin saturated in TuMV inoculum. Plants were maintained at 18°C, 10-hour day-length for the duration of the experiment.

Assessment of infection

Plants were visually assessed every two days after inoculation for a period of 20 days. Inoculated and uninoculated leaves were sampled for ELISA following the order of the randomisation of blocks (Figure 2.1):
four days post-inoculation, leaves were taken from plants in modules 1, 9 & 14

eight days post-inoculation, leaves were taken from plants in modules 5, 8 & 11

twelve days post-inoculation, leaves were taken from plants in modules 4, 7 & 13

sixteen days post-inoculation, leaves were taken from plants in modules 3, 10 & 15

twenty days post-inoculation, leaves were taken from plants in modules 2, 6 & 12

Individual leaves were ground using ceramic tiles and pestles. ELISA was carried out as described by Jenner & Walsh (1996), with changes described in section 2.2.

2.3.3 Results

Symptoms were classified as systemic chlorosis, leaf distortion, purple veins and mottling. No obvious symptoms were seen in any of the inoculated leaves. ELISA testing showed that 100% infection was achieved using muslin to inoculate the plants (Table 2.2). Virus replication was detected just four days

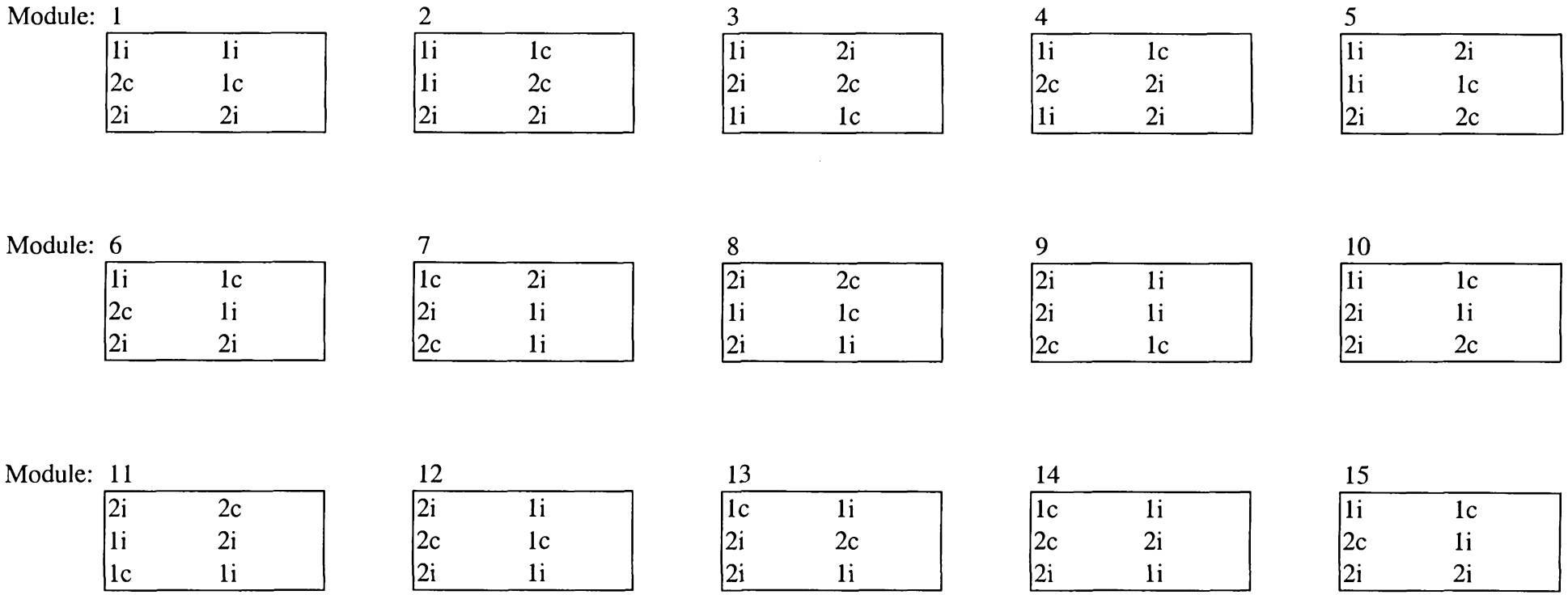


Figure 2.1 Randomised block design for an experiment to investigate the improvement of inoculation technique of *A. thaliana* with turnip mosaic virus. 1, *A. thaliana* ecotype col-*ndr 1*; 2, *A. thaliana* ecotype col-γ 1-2; i, inoculated plant; c, uninoculated plant (control).

Table 2.2 Results of experiment to investigate the improvement of the technique to mechanically inoculate *A. thaliana* with turnip mosaic virus (TuMV) and the technique to assess level of TuMV infection.

Days post inoculation	Modules	% plants with symptoms	% leaves TuMV +ve in ELISA	
			inoculated	uninoculated
4	1, 9, 14	0	100	36
8	5, 8, 11	6	100	27
12	4, 7, 13	100	100	75
16	3, 10, 15	100	100	75
20	2, 6, 12	100	100	100

after inoculation in 100% of inoculated leaves and 36% of uninoculated leaves, even though none of the plants showed symptoms. ELISA tests carried out eight days after inoculation gave similar results to those at four days after inoculation. By 12 days post-inoculation systemic infection was detected in 75% of plants. All plants that were tested 20 days after inoculation showed symptoms, described previously, in inoculated and uninoculated leaves. TuMV was also detected by ELISA in the inoculated and uninoculated leaves of all plants 20 days post-inoculation. None of the controls had symptoms and all were negative in ELISA.

2.3.4 Discussion

Inoculating with muslin gave 100% infection so no further inoculation methods were evaluated. This method is preferential to other methods because it is also used in *Brassica* experiments (see Chapters 3 and 4) and hence results obtained with *A. thaliana* can be directly compared to *Brassica*.

The results from this experiment showed that visual assessment is only necessary once a week because symptoms do not change dramatically day-to-day. Results showed that three weeks after inoculation ELISA detection of TuMV was consistent with symptoms observed, with 100% TuMV infection detected by ELISA in inoculated and uninoculated leaves being coincident with 100% symptom expression. In future experiments symptoms will be assessed visually every week for a period of three weeks at the end of which ELISA will be carried out on inoculated and uninoculated leaves. It is important to sample both inoculated and uninoculated leaves in order to identify the R phenotype that has been seen in *Brassica* where replication of virus can be detected in inoculated leaves but not in uninoculated leaves (Jenner & Walsh, 1996). This form of resistance could be misclassified as susceptible to systemic infection if whole plants were to be sampled rather than individual leaves. These procedures for visual assessment and ELISA testing are very similar to those used in virus testing *Brassica* spp., which makes comparison of results appropriate.

2.4 SCREENING *A. THALIANA* FOR RESISTANCE TO TUMV

2.4.1 Introduction

Optimum temperature, method of inoculation, time intervals for visual assessment and ELISA have been identified. Using these conditions it will now be possible to assess the interaction between parents of different *A. thaliana* mapping populations and five TuMV isolates. The availability of the mapping populations means that if resistance is identified in any of the parents it will be possible to map any gene(s) that confer resistance relatively rapidly.

2.4.2 Materials & Methods

Plant material

Seed of ecotypes Col-5, Nd-1, Ler-1, Ler-*ttg*, Ws-3, Oy-0, Wei-1, Ksk-1, Ksk-2, Col-*ndr1*, Col-0 and Col-γ 1-2 used in this study were obtained from E. Holub, HRI, Wellesbourne, UK.

Virus Isolates

TuMV isolates UK 1, CZE 1, CHN 5 and CDN 1 belonging to pathotypes 1, 3, 3 and 4 respectively (Jenner & Walsh, 1996) were used in these experiments. These were chosen because they have been used in screening *B. rapa* and *B. napus* lines for resistance (see Chapter 3). JPN 1, a member of pathotype 7 (Jenner & Walsh, 1996), was chosen because it is a less virulent isolate than the other four on *Brassica*, so may increase the chance of identifying resistance in *A. thaliana.*

Inoculation technique

Plants were grown as described in section 2.2. At four weeks old, three carborundum-dusted leaves were marked with a glass pasteur pipette, these leaves were inoculated with virus inoculum, prepared as described by Jenner & Walsh (1996), using muslin and maintained at 18°C for the duration of the experiment. Five plants of each line were inoculated with each virus isolate. Separate experiments were carried out for each virus isolate in order to eliminate any risk of cross-contamination. Controls were treated as described in section 2.2.

Assessment of Infection

Plants were assessed visually every week for three weeks. Three weeks after inoculation inoculated leaves and uninoculated leaves were sampled for ELISA; ELISA conditions were as described by Jenner & Walsh (1996) with changes described in section 2.2.

2.4.3 Results

All plants of all lines tested were susceptible to all isolates used. Systemic chlorosis, leaf distortion, purple veins and mottling were the only symptoms seen. Necrosis was not observed in any plant line. Figure 2.2 shows typical phenotypes when *A. thaliana* is infected with UK 1 and CZE 1 under these conditions. Figure 2.2 also shows that TuMV isolate UK 1 (pathotype 1) produces a more severe effect on *A. thaliana* ecotypes than TuMV isolate CZE 1 (pathotype 3).

2.4.4 Discussion

This experiment has shown that there is no resistance in any of the *A. thaliana* ecotypes to any of the five TuMV isolates used. This may be due to the particular lines selected and may not be the case for all *A. thaliana* lines. The differences

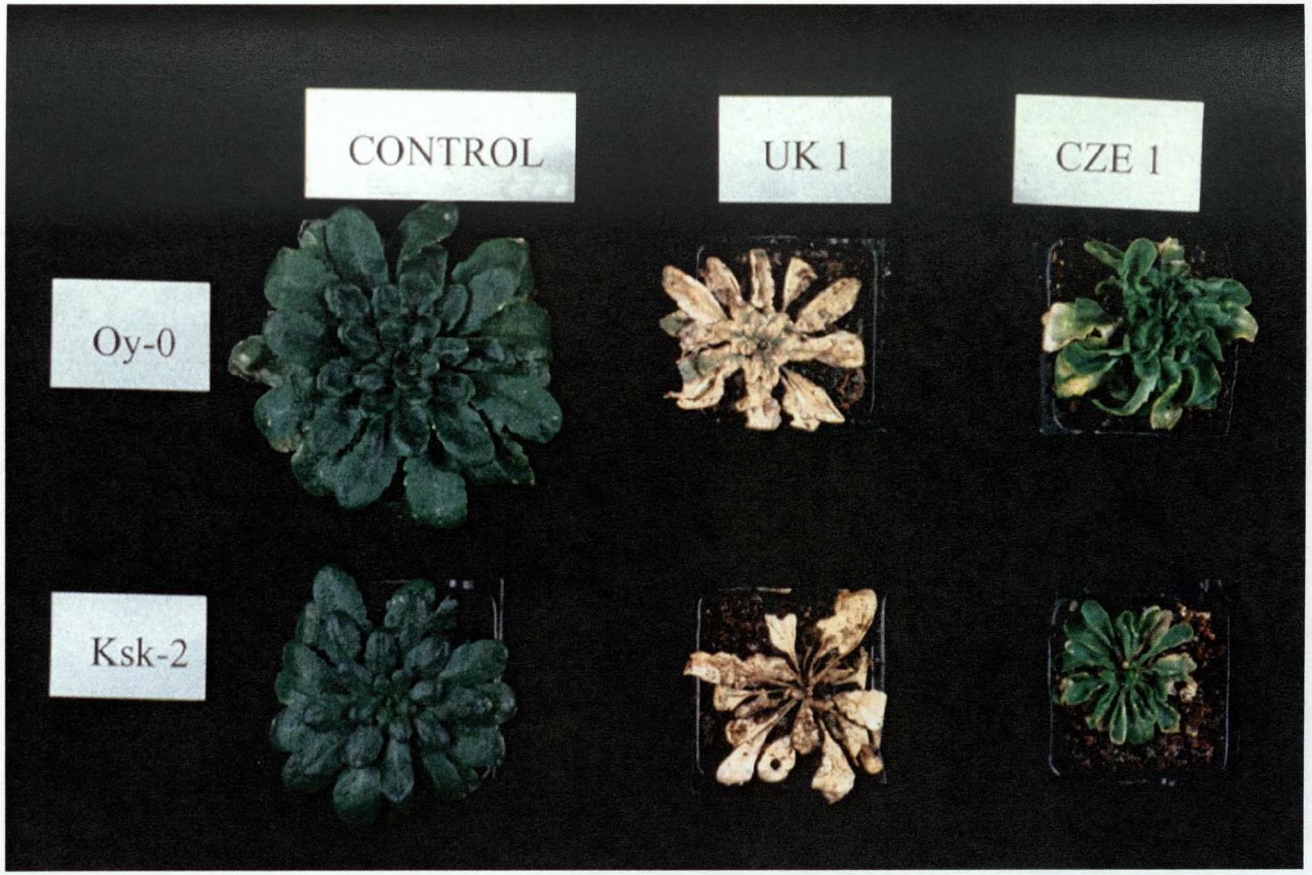


Figure 2.2 Comparison of uninfected (control) plants from *A. thaliana* ecotypes Oy-0 and Ksk-2 with plants from the same ecotypes infected with TuMV isolates UK 1 and CZE 1.

observed in severity of symptoms (Figure 2.2) suggest that different interactions are occurring between TuMV isolates and different *A. thaliana* lines. Further investigation of TuMV-*A. thaliana* interactions, perhaps using a TuMV isolate labelled with GUS (F. Ponz, personal communication), may lead to a better understanding of these complex mechanisms.

A source of TuMV resistance previously identified in *A. thaliana*, Bay-0, (Martin-Martin *et al.*,1999) has been shown to be environmentally unstable with loss of resistance when temperature and day length were altered (F. Ponz & F. Sanchez, personal communication). Martin-Martin *et al.* (1999) inoculated plants once they had bolted. In my experiment the plants that were inoculated had not bolted. When I tested Bay-0 for resistance to UK 1 under the same conditions described in the Materials & Methods section of this chapter, it was susceptible (data not shown). The more advanced developmental stage of the plants used by Martin-Martin *et al.* (1999) may have had an effect on resistance due to expression of different genes at a later stage of plant development. Leisner *et al.* (1993) observed that some early-flowering *A. thaliana* ecotypes were resistant to systemic infection by cauliflower mosaic virus. If the development of early flowering plants was retarded by suboptimal growth conditions these 'resistant' ecotypes became susceptible (Leisner *et al.*,1993).

A more promising avenue of investigation may be to test wild *A. thaliana* ecotypes which are probably more heterogeneous than the inbred lines tested in my experiments. Alternatively, the wide variety of mutants that are available could be screened. *A. thaliana* has already proved to be a useful model for other plant-pathogen interaction studies, for example *Pseudomonas syringae* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995), *Peronospora parasitica* (Parker, *et al.*, 1993; Parker *et al.*, 1997; Bittner-Eddy *et al.*, 1999; Bittner-Eddy *et al.*, 2000) and TEV (Mahajan *et al.*, 1998; Chisholm *et al.*, 2000; Whitham *et al.*, 2000). If resistance to TuMV could be identified in *A. thaliana,* that is not dependent on environment, major advances in knowledge about TuMV-plant interactions could be achieved.

CHAPTER 3

IDENTIFICATION OF RESISTANCE TO TuMV IN *BRASSICA RAPA* AND *B. NAPUS*

3.1 INTRODUCTION

Brassica rapa and *B. napus* lines from Asia, USA and UK were screened for resistance to TuMV isolates representing the three most common pathotypes in Europe, pathotypes 1, 3 and 4 (Jenner & Walsh, 1996). Some of the *Brassica* lines had previously been identified as TuMV resistant and were included in this study for further characterisation. The main aim of this work was to identify *Brassica* lines that had resistance to all four TuMV isolates and/or those that had new specificities of interaction.

Provvidenti (1980) developed a TuMV classification system based on interaction of isolates with several *B. rapa* lines. Isolates were classified into strains C1, C2, C3, C4 (Provvidenti, 1980) and C5 (Green & Deng, 1985). Jenner & Walsh (1996) grouped TuMV isolates into 12 pathotypes using four *B. napus* differentials (Table 1.3, Chapter 1). They found that a TuMV isolate representing strain C1 belonged to pathotype 1 and isolates representing C2-C5 belonged to pathotype 3. The classification of C2-C5 into pathotype 3 suggests that this pathotyping system would benefit from the introduction of host plant lines that are able to differentiate between pathotype 3 isolates. Several lines used by Provvidenti (1980), or selections from these lines, were included in this study to compare results with Provvidenti (1980) and to find out if they were able to differentiate between pathotype 3 isolates.

3.2 MATERIALS & METHODS

3.2.1 Plant Material

The origins of the *B. rapa* and *B. napus* lines characterised in this study are shown in Table 3.1. Lines P63 PI418959, PI418957C and 0-2 plant 5 are selfed progeny of plants identified during previous tests for resistance on lines PI418959, PI418957 and 0-2 respectively (J.A. Walsh & D.J. Lydiate, personal communication).

3.2.2 Virus isolates

The TuMV isolates used in resistance testing were UK 1, CZE 1, CDN 1 and CHN 5 (Table 3.2). The first three isolates were used because they are the type members of pathotypes 1, 3 and 4 respectively (Jenner & Walsh, 1996). CHN 5 was used because it is representative of an aggressive TuMV strain, C5, from Taiwan and China (Green & Deng, 1985; Liu *et al.*, 1990).

3.2.3 Plant growth conditions

Seeds were sown in 7cm pots in M2 peat compost (Fisons, UK) and grown in an insect proof glasshouse at 18°C for the duration of the experiment.

3.2.4 Virus propagation and maintenance of isolates

Virus isolates were maintained in a susceptible host *B. juncea* cultivar (cv.) Tendergreen (TGM) by mechanical inoculation (Jenner & Walsh, 1996), in an insect-proof glasshouse at 18°C.

Table 3.1 Sources of *Brassica rapa* and *B. napus* lines used in testing for resistance to turnip mosaic virus.

Plant Line	Plant Type	Source
NVRS 4680	*Brassica rapa* (Chinese cabbage)	Genetic Resources Unit, Horticulture Research International, Wellesbourne, UK
NVRS 5892	*Brassica rapa* (Chinese cabbage)	
NVRS 5965	*Brassica rapa* (Chinese cabbage)	
NVRS 6143	*Brassica rapa* (Chinese cabbage)	
NVRS 6202	*Brassica rapa* (Chinese cabbage)	
IHR3111	*Brassica rapa* (Chinese cabbage)	[1] with kind permission from J. E. Bradshaw, SCRI (all are inbred lines derived from cultivars or crosses)
IHR6165	*Brassica rapa* (Chinese cabbage)	
HRI5581	*Brassica rapa* (Chinese cabbage)	
IHR7581	*Brassica rapa* (Chinese cabbage)	
HRI12832	*Brassica rapa* (Chinese cabbage)	
Bangholm Wilby[1]	*Brassica napus* (Swede)	
Bangholm Ruta Otofte[1]	*Brassica napus* (Swede)	
Ne Plus Ultra[1]	*Brassica napus* (Swede)	
Bangholm Dima x Melfort[1]	*Brassica napus* (Swede)	
Emerald King[1]	*Brassica napus* (Swede)	
Bangholm Magres x Champion (DBO 15a)[1]	*Brassica napus* (Swede)	
Vogesa[1]	*Brassica napus* (Swede)	
Scotia[1]	*Brassica napus* (Swede)	
Magnificent[1]	*Brassica napus* (Swede)	
Vogesa x Marian[1]	*Brassica napus* (Swede)	
0-2	*Brassica rapa* (Chinese cabbage)	Asian Vegetable Research and Development Center, Taiwan
BP058		
Jong Bai No. 1	*Brassica rapa* (Chinese cabbage)	Vegetable & Flower Research Institute/Chinese Academy of Agricultural Sciences, China
Jong Bai No. 2	*Brassica rapa* (Chinese cabbage)	
Jong Bai No. 4	*Brassica rapa* (Chinese cabbage)	
Jong Bai No. 13	*Brassica rapa* (Chinese cabbage)	
Shern Yang	*Brassica rapa* (Chinese cabbage)	Shern Yang Academia of Agricultural Sciences, China
Hybrid M4	*Brassica rapa* (Chinese cabbage)	Fu-Jiaw Academia of Agricultural Sciences, China
Sin Kong Bin 65	*Brassica rapa* (Chinese cabbage)	
Jin G 55	*Brassica rapa* (Chinese cabbage)	Tien-Jin Academia of Agricultural Sciences, China
Jin G 75	*Brassica rapa* (Chinese cabbage)	
Jin G 80	*Brassica rapa* (Chinese cabbage)	
PI419105	*Brassica rapa* (Chinese cabbage)	R. Provvidenti, Cornell University, USA
PI391560	*Brassica rapa* (Chinese cabbage)	
Crusader	*Brassica rapa* (Chinese cabbage)	
PI418957	*Brassica rapa* (Chinese cabbage)	
PI418959	*Brassica rapa* (Chinese cabbage)	
Cobra 22S[2]	*Brassica napus* (Oilseed rape)	Horticulture Research International, Wellesbourne, UK [2] J. Vicente [3] N. Cogan
Cobra 14R[2]	*Brassica napus* (Oilseed rape)	
Cobra 13R[2]	*Brassica napus* (Oilseed rape)	
Bristol[3]	*Brassica napus* (Oilseed rape)	
Capricorn[3]	*Brassica napus* (Oilseed rape)	
Apex[3]	*Brassica napus* (Oilseed rape)	
Global[3]	*Brassica napus* (Oilseed rape)	

Table 3.2 Turnip mosaic virus isolates used in resistance testing of *Brassica rapa* and *B. napus* lines.

Virus isolate	Origin	Differential lines				Pathotype	References
		R4	S1	165	S6		
UK 1	UK	0	+	0	+	1	Walsh (1989); Jenner & Walsh (1996); Lehmann *et al.* (1997); Walsh *et al.* (1999); Jenner *et al.* (2000)
CZE 1	Czech Republic	$+_N$	+	0	+	3	Jenner & Walsh (1996); Lehmann *et al.* (1997); Walsh *et al.* (1999)
CHN 5	Taiwan and China	$+_N$	+	0	+	3	Green & Deng (1985); Liu *et al.*, (1990); Jenner & Walsh (1996); Walsh *et al.* (1999)
CDN 1	Canada	+	+	+	+	4	Walsh (1989); Jenner & Walsh (1996); Lehmann *et al.* (1997); Walsh *et al.* (1999)

Notes:
- 0 Apparent immunity; no symptoms observed in inoculated or uninoculated leaves, no virus detected by ELISA or back inoculation.
- \+ Susceptibility; chlorotic symptoms observed in inoculated and uninoculated leaves, virus detected by ELISA.
- $+_N$ Susceptibility; necrotic symptoms observed in inoculated and uninoculated leaves, virus detected by ELISA.

To prevent cross contamination of isolates the following precautions were taken:

- Latex gloves and muslin were discarded and hands were washed after working on plants infected with a particular isolate before proceeding to plants infected with another isolate.
- Plants inoculated with different isolates were isolated by distance.

3.2.5 Inoculation Procedure

Virus inocula were prepared by grinding 1g of systemically infected *B. juncea* leaves per 2ml of cold inoculation buffer (1% K_2HPO_4, 0.1% Na_2SO_3) as described by Jenner & Walsh (1996). Plants were inoculated at the two to three true-leaf stage (approx. 21 days post planting). Leaves were dusted with carborundum (300 mesh) and then rubbed with a muslin pad saturated with virus inoculum (Jenner & Walsh, 1996). Inoculated plants were maintained in an insect proof glasshouse at 18°C. Four to ten plants were inoculated per virus isolate per line depending on the availability of seed.

The integrity of isolates was checked by mechanical inoculation to the four *B. napus* differential lines, R4, S6, 165 (Walsh, 1989; Jenner & Walsh, 1996) and S1 (Jenner & Walsh, 1996) on every occasion that experimental plants were inoculated.

3.2.6 Assessment of infection

Symptoms were recorded by visual inspection at weekly intervals up to 4 weeks after inoculation. At the end of the 4-week period inoculated and uninoculated leaves were tested for the presence of TuMV using indirect plate-trapped antigen (PTA) ELISA. Conditions used for ELISA were similar to those described by Jenner & Walsh (1996). Samples were ground and the sap was diluted 1:10 in coating buffer (Na_2CO_3, 1.6g.l^{-1}, $NaHCO_3$, 30g.l^{-1}). 100µl of the diluted sap was loaded into duplicate wells on a microtitre plate. After an overnight incubation at

4°C the plates were washed 3 times for 3 minutes in phosphate buffered saline ($Na_2HPO_4.12H_2O$, 2.9g.l^{-1}; KH_2PO_4, 0.2g.l^{-1}; NaCl, 8g.l^{-1}; KCl, 0.2g.l^{-1}) containing 0.5% Tween-20 (PBS-T, pH7.3). The plates were then coated with TuMV monoclonal antibody EMA67 (Jenner *et al.*, 1999), diluted 1/2500 in PBS-T containing 0.05% BSA and incubated at room temperature for 2 hours. After incubation, the plates were washed in PBS-T (as described above) and coated with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma A-3562), diluted 1/5000 in PBS-T containing 0.05% BSA. The plates were incubated for a further 2 hours at room temperature. After incubation the plates were washed (as described above) and the colour reaction developed by adding 100µl substrate buffer (diethanolamine, 97ml.l^{-1} pH9.8) to each well. The reactions were read with a Titertek Multiskan MCC/340 plate reader at an absorbance of 405nm. In all ELISA tests the healthy controls were used as blanks and a reaction was considered positive if the absorption value was above 0.300.

3.3 RESULTS

3.3.1 Classification of phenotypes

Phenotypes were classified into three types, as described in Jenner & Walsh (1996): apparent immunity (0), resistance (R) and susceptibility (+ or $+_N$) (Tables 3.3 and 3.4). Apparent immunity (0) is defined as no symptoms following weekly visual assessments and no virus detected in inoculated or uninoculated leaves 3-4 weeks after inoculation using ELISA and back inoculation. Resistance is defined as the presence of chlorotic spots (R) in inoculated leaves, but no symptoms and no virus detected in uninoculated leaves using ELISA and back inoculation, i.e. resistance to systemic spread of TuMV. The susceptibility phenotype is defined by the presence of visual symptoms, chlorotic spots and/or mosaic (+) or necrosis ($+_N$) as well as positive results in ELISA tests on inoculated and uninoculated leaves.

3.3.2 Integrity of isolates

All isolates used in this study induced reactions similar to those described by Jenner & Walsh (1996) in line with their pathotypic specificity on the four *B. napus* lines in all experiments (Table 3.2).

3.3.3 *B. rapa*

Fourteen of the 28 *B. rapa* lines tested were resistant to all four isolates used (Table 3.3). Eleven of these differentiated the pathotype 3 isolates, CZE 1 and CHN 5. IHR 3111, PI419105, PI391560, P63 PI418959 and BP058-4 segregated in their interaction with one or both of the pathotype 3 isolates and thus have the potential to discriminate between them. HRI 12832 had an unusual specificity of interaction because it had apparent immunity to the pathotype 1 isolate and some

Table 3.3 Specificity of interaction of *Brassica rapa* lines with four turnip mosaic virus isolates.

Plant Line	Virus Isolate (Pathotype)			
	UK 1 (1)	CZE 1 (3)	CHN 5 (3)	CDN 1 (4)
NVRS 5892	+	+	+	+
NVRS 6143	+	+	+	+
NVRS 6202	+	+	+	+
Crusader	+	+	+	+
IHR 7581	+	+	+	+
NVRS 5965	+/+$_N$	+	+	+
IHR 6165	+/+$_N$	+	+	+
NVRS 4680	+$_N$	+	+	+
IHR 3111	+$_N$	+/+$_N$	+	+
HRI 5581	0	+$_N$	+$_N$	+
PI419105	0*/+	0/+	0/+	+
HRI 12832	0	+$_N$	+$_N$	R/+
PI391560	0/+	0/R/+	+	0/R/+
Jong Bai No. 1	0	0*	R	R
Jong Bai No. 2	0	0*	R	R
Jong Bai No. 13	0	0	R	R
Hybrid M4	0	0	R	R
Sin Kong Bin 65	0	0	R	R
JinG75	0	0	R	R
JinG80	0	0	R	R
Jong Bai No. 4	0/R	0	R	R
P63 PI418959	0	0*	0/R	R
0-2 plant 5	0	0	R	0*/R
PI418957C	0	0	R	0*/R
Shern Yang	0	0	R	0/R
BP058-4	0/R	0/R	0/R	0*/R
JinG55	R	R	R	R*

Notes:
- 0 Apparent immunity; no symptoms observed in inoculated or uninoculated leaves, no virus detected by ELISA or back inoculation.
- R Resistance; chlorotic symptoms observed in inoculated leaves, virus detected by ELISA; no symptoms observed in uninoculated leaves and no virus detected by ELISA or back inoculation.
- + Susceptibility; chlorotic infection observed in inoculated and uninoculated leaves, virus detected by ELISA.
- +$_N$ Susceptibility; necrotic infection observed in inoculated and uninoculated leaves, virus detected by ELISA.
- * Selected for crosses for analysis of genetic inheritance of TuMV resistance

Table 3.4 Specificity of interaction of *Brassica napus* lines with four turnip mosaic virus isolates.

Plant Line	Virus Isolate (Pathotype)			
	UK 1 (1)	CZE 1 (3)	CHN 5 (3)	CDN 1 (4)
Bristol	+	+	+	+
Capricorn	+	+	+	+
Bangholm Ruta Otofte	0	+	+	+
Ne Plus Ultra	0	+	+	+
Emerald King	0	+	+	+
Bangholm Dima x Melfort	0	+	+	+
14R	0	$+_N$	$+_N$	+
Bangholm Wilby	0	$+_N$	$+_N$	+
13R	0/+	+	$+/+_N$	+
Apex	+	+	R/+	+
22S	$+_N$	$+_N$	$+_N$	0*
Magnificent	0/R	R/+	+	+
Global	0	$R/+_N$	$+_N$	+
Vogesa x Marian	0	0	R	+
Vogesa	0	0/+	0/R	+
Scotia	0	R/+	0/R/+	+
DBO15a	0/+	0/R	0/R	+

Notes: 0 Apparent immunity; no symptoms observed in inoculated or uninoculated leaves, no virus detected by ELISA or back inoculation.

R Resistance; chlorotic symptoms observed in inoculated leaves, virus detected by ELISA; no symptoms observed in uninoculated leaves and no virus detected by ELISA or back inoculation.

\+ Susceptibility; chlorotic infection observed in inoculated and uninoculated leaves, virus detected by ELISA.

$+_N$ Susceptibility; necrotic infection observed in inoculated and uninoculated leaves, virus detected by ELISA.

* Selected for crosses for analysis of genetic inheritance of TuMV resistance

plants were resistant to systemic movement of the pathotype 4 isolate but all plants were susceptible to both of the pathotype 3 isolates (Table 3.3).

3.3.4 *B. napus*

The most common specificity of resistance observed in the *B. napus* lines tested was single pathotype apparent immunity (0) or resistance (R) (Table 3.4). Seven of the nine lines with single pathotype resistance were apparently immune to UK 1. Only 22S had apparent immunity to the pathotype 4 isolate, CDN 1 (Figure 3.1). Apex had a proportion of plants that were resistant to CHN 5. Two lines were susceptible to all isolates. The other six lines tested showed some resistance to pathotype 1 and both pathotype 3 isolates but were susceptible to the pathotype 4 isolate (Table 3.4). The line Vogesa x Marian could differentiate between the pathotype 3 isolates because it has apparent immunity to CZE 1 and resistance to systemic movement of CHN 5 (Table 3.4). Lines 13R, Apex, Magnificent, Global, Vogesa, Scotia and DBO15a have potential to differentiate between the pathotype 3 isolates (Table 3.4).



Figure 3.1 *Brassica napus* line 22S inoculated with turnip mosaic virus isolates CDN 1 and UK 1. The plant on the left hand side is apparently immune to CDN 1; plant on the right hand side is susceptible, with stunting and necrotic symptoms, to UK 1.

3.4 DISCUSSION

Thirty-three sources of resistance to TuMV in *B. rapa* and *B. napus* have been identified in this study, including lines with specificities of interaction that had not been encountered before. Several of these lines plus other lines that were susceptible to all four isolates have the potential to increase the resolution of the pathotyping system previously described by Jenner & Walsh (1996).

The results from lines included in my study that were used by Provvidenti (1980) has provided some useful comparisons and information about their interaction with UK 1, CZE 1, CHN 5 and CDN 1. Results from my study for lines Crusader, PI391560, P63 PI418959 and PI418957C were similar to those found for the respective lines tested by Provvidenti (1980); Green & Deng (1985) also found similar results for Crusader. In contrast, I found different results for line PI419105 to those found by Provvidenti (1980). In my study it segregated for apparent immunity and susceptibility to both UK 1 and CZE 1 whereas it was found to be uniformly susceptible to C1 and C4 but was uniformly resistant to C2 and C3 by Provvidenti (1980). My results for PI418957C are also different to those found by Green & Deng (1985), they found the line was susceptible to C5 whereas I found it to be resistant to systemic movement of CHN 5. The differences between my results and previous results may be explained by the fact that some lines tested were selfed progeny from individual plants. Also some of the seed I received had been produced by bulking selfs of more than one plant of the Provvidenti (1980) lines. For example, PI419105 may have been heterozygous for a recessive resistance gene to C1 when tested by Provvidenti (1980) giving a susceptible phenotype. Following subsequent selfing it would be expected that the line would segregate for some form of resistance and susceptibility to pathotype 1, as observed in my results (Table 3.3), because pathotype 1 is comparable to C1 (Jenner & Walsh, 1996). The differences between results could also be due to the use of different isolates and/or different inoculation and assessment conditions to those used by Provvidenti (1980) and Green & Deng (1985).

The comparison of these studies highlights the need to expand the pathotyping system by producing further characterised host plant differential lines. Eleven *B. rapa* lines, including PI418957C, and one *B. napus* line reliably differentiate the pathotype 3 isolates. Several *B. rapa* and *B. napus* lines have potential to differentiate between the pathotype 3 isolates, including three described by Provvidenti (1980), PI419105, PI391560 and P63 PI418959. If homozygous lines can be produced from these they could be incorporated into the pathotyping system described by Jenner & Walsh (1996) that I have been using. This would give better resolution of pathogenicity and more detailed information on isolates.

Pathotype 4 isolates infect all of the *B. napus* differential lines used in the Jenner & Walsh (1996) pathotyping system. The 22S line provides the first example of apparent immunity to CDN 1 in *B. napus*. It could give further resolution as a differential in that it might differentiate between isolates currently designated as pathotypes 1-12 (Jenner & Walsh, 1996).

The broad-spectrum resistance in the line 0-2 appears to be typical of most of the *B. rapa* lines tested in this study; 0-2 was also found to be resistant to strains C1-C5 by Anon (1990), Yoon *et al.* (1993) and Suh *et al.* (1996). In contrast, no broad-spectrum resistance has been found in *B. napus* in this study; *B. napus* lines were susceptible to all pathotypes or resistant to only one or two pathotypes. *B. napus* is an amphidiploid of *B. rapa* and *B. oleracea*. The presence of the *B. oleracea* genome may affect the expression of resistance in *B. napus* because only partial resistance has been identified in *B. oleracea* (Walkey & Pink, 1988; Pink & Walkey, 1990). The high frequency of broad-spectrum resistance in *B. rapa* and lack of it in *B. napus* could also be due to differences in breeding strategies or an artefact of the selection of lines for this study.

In the majority of *B. napus* lines I tested, UK 1 (pathotype 1) replication could not be detected. Tomlinson & Ward (1982), Walsh (1989) and Walsh *et al.* (1999) have also described apparent immunity to UK 1 in *B. napus*. *B. napus* has been grown in Europe for centuries (Fussell, 1955) and pathotype 1 isolates are the

most common isolates in Europe (Jenner & Walsh, 1996). This suggests that *B. napus* has been under selection pressure for resistance to members of pathotype 1. All lines with resistance to pathotype 3 are also resistant to pathotype 1, except Apex, which has some resistance to systemic movement of CHN 5 but is susceptible to UK 1. The rare occurrence of resistance to pathotype 3 isolates without resistance to pathotype 1 isolates suggests that *B. napus* may not encounter pathotype 3 isolates without encountering pathotype 1 isolates. Alternatively, resistance to pathotype 1 isolates could be tightly linked to resistance to pathotype 3 isolates or the same gene could control resistance to both pathotypes. Walsh (1989) and Jenner & Walsh (1996) have also described immunity to both pathotype 1 and 3 isolates in the differential line 165. Pathotype 3 isolates are able to overcome the recently mapped TuMV resistance gene, *TuRB01*, which is effective against pathotype 1 isolates (Walsh *et al.*, 1999). The mutation of UK 1 (pathotype 1) to UK 1M (pathotype 3) (Lehmann *et al.*, 1997) suggests that pathotype 3 isolates could be derived from pathotype 1 isolates. If TuMV and brassicas have a gene-for-gene type of relationship, and the assumption that there are few differences between pathotype 1 and 3 isolates is correct, variants of genes that confer resistance to pathotype 1 isolates may confer resistance to pathotype 3 isolates. This might suggest that *TuRB01* (Walsh *et al.*, 1999) could be derived from or be the precursor of resistance gene(s) to pathotype 3 isolates.

The low frequency of resistance/apparent immunity to the pathotype 4 isolate, CDN 1, suggests that *B. napus* might only have recently encountered isolates of this pathotype. It also suggests that pathotype 4 isolates are not derived from, or are not closely related to, pathotype 1 or 3 isolates.

The susceptibility phenotypes suggest that the plants provided all the necessary prerequisites for the virus to accomplish replication in the initially infected cell, cell-to-cell movement and finally long distance movement of the virus to sites distal to infection foci. The necrotic susceptibility phenotype kills plants quickly compared to the mosaic susceptibility phenotype. This suggests that host cell

death is occurring rapidly and thus restricting pathogen movement. This could be a type of defence response, related to the hypersensitive response, that is occurring throughout the plant. Evidence to support this comes from the resistance gene *TuRB01* which produces an extreme form of resistance against TuMV isolate UK 1, perhaps a type of hypersensitive response (Walsh *et al.*, 1999). When this resistance is overcome by a mutant of UK 1, UK 1M, a severe necrotic reaction occurs and the plants die (Lehmann *et al.*, 1997) which suggests that the *TuRB01* defence response may be activated but it occurs throughout the plant.

In the resistance to systemic movement phenotype, replication must occur and the virus is likely to accomplish some cell-to-cell movement in the infected leaf but it is prevented either from achieving extensive cell-to-cell movement and/or from entering the vascular system and moving to leaves distal to infection foci. This restriction could occur by a mechanism that prevents virus entry into the phloem or, less likely, the virus is able to enter the phloem and move through the vasculature but is unable to enter distal leaves. This type of resistance to systemic movement of virus has been identified in *A. thaliana* ecotypes against TEV (Mahajan *et al.*, 1998). Chisholm *et al.* (2000) and Whitham *et al.* (2000) suggested that the gene products from *RTM1* and *RTM2* interact with the complex formed by the capsid protein, CI and HC-Pro to prevent virus movement. This scenario may also apply to the resistance to systemic movement of TuMV I have identified in a number of *B. rapa* and *B. napus* lines.

The apparent immunity phenotype suggests that after initial infection there is no viral replication, or that replication occurs at a level below the limits of detection of the ELISA and back-inoculation techniques used. Extreme forms of resistance have been identified to TuMV (Walsh, 1989; Walsh *et al.*, 1999; Rusholme, 2000) and potato virus X (PVX) (Baulcombe *et al.*, 1994). The *Rx* gene confers an extreme form of resistance to PVX that is epistatic to a hypersensitive response and it controls separate virus resistance and cell death responses (Bendahmane *et al.*, 1999). Another example of extreme resistance without occurrence of the

hypersensitive response has been observed in the *A. thaliana dnd1* mutant that still exhibits a gene-for-gene type restriction of *Pseudomonas syringae* growth even though it has lost its ability to produce a hypersensitive response (Yu *et al.*, 1998). This suggests that the hypersensitive response may have a secondary role in restricting pathogen growth rather than an essential role (Yu *et al.*, 1998). It is possible that the type of resistance occurring in plants with apparent immunity to TuMV may be similar to that conferred by *Rx* against PVX. Further investigations will have to be carried out to discover how the resistance mechanisms operate. The production of an infectious clone of TuMV expressing β-glucuronidase (GUS) or jellyfish green fluorescent protein (GFP) (F. Ponz & F. Sanchez, personal communication) will facilitate further investigations into mechanisms of resistance.

A number of the *B. rapa* and *B. napus* lines have been taken forward for genetic characterisation, identification of markers linked to the resistance gene(s) and mapping of such gene(s). To eventually produce broad-spectrum TuMV resistance it will be necessary to identify combinations of resistance genes that will provide resistance to all TuMV isolates.

CHAPTER 4

CHARACTERISATION OF THE GENETIC BASIS OF TuMV RESISTANCE IN *BRASSICA RAPA* & *B. NAPUS*

4.1 INTRODUCTION

To characterise the genetic basis of TuMV resistance, several lines were selected from those described in Chapter 3. These included *B. rapa* lines PI418957C, PI418959, PI419105, Jin G 55, BP058-4, 0-2, Jong Bai No. 1 and Jong Bai No. 2. The lines were chosen because their interactions with TuMV pathotypes suggested they either were, or had the potential to be, resistant or apparently immune to the three most prevalent TuMV pathotypes (see Chapter 3, Table 3.3). Furthermore, it was previously established that lines PI418957C, 0-2, Jong Bai No. 1 and Jong Bai No. 2 were able to differentiate between the pathotype 3 isolates, CHN 5 and CZE 1 and lines PI418959 and PI419105 had the potential to differentiate between them (Table 3.3). The *B. napus* line 165 was chosen for further characterisation because it has broad-spectrum resistance to pathotypes 1-3, 5-7 and 9-11 (see Chapter 1, Table 1.3; Jenner & Walsh, 1996) and has already been partially characterised by Shattuck & Stobbs (1987) who found a single dominant allele for TuMV resistance. Line 22S is the first example, in *B. napus*, of resistance to pathotype 4 (see Chapter 3, Table 3.4). It is important to characterise the genetic basis of TuMV resistance in line 22S because pathotype 4 isolates have overcome all other resistance sources previously identified in *B. napus*. Line 22S could therefore be a very useful source of effective TuMV resistance.

Two forms of resistance were described in Chapter 3: R, resistance to systemic spread of the virus and 0, apparent immunity, where no virus could be detected. To simplify the language used in this chapter and in Chapter 5, both forms of resistance will be referred to as "resistance."

4.2 MATERIALS AND METHODS

4.2.1 Plant Material

Lines selected for characterisation of the genetic inheritance of resistance are listed in Table 4.1.

Production of S_1, F_1, F_2 and B_1 generations

Self-incompatibility is common in *B. rapa*. To produce selfed generations (S_1) from TuMV resistant *B. rapa* plants, denoted by * in Table 3.3 (Chapter 3), selfing by bud-pollination was employed. *B. rapa* lines 0-2, PI418959 and PI419105 were highly self-incompatible so were treated with a 5% NaCl solution 2 hours before bud-pollination was attempted. Unopened flower buds were opened with tweezers and the stigma rubbed with pollen from the stamen of an open flower from the same plant. Each flower bud was labelled with the date it was pollinated and pollinated flower buds were covered with cellophane "bread bags" to prevent cross-pollination. Once the seed-pods had ripened (approx. 8 weeks after the last bud was pollinated), they were dried, harvested and the seed collected.

The TuMV resistant *B. napus* plants, denoted by * in Table 3.4, were self-compatible so did not require bud-pollination. Cellophane "bread bags" were placed over inflorescences at the unopened bud stage to prevent cross-pollination; each plant was labelled with the date it was bagged. As buds opened the plants were shaken gently to encourage transfer of pollen from stamen to stigma. Once the seed-pods had ripened and dried they were harvested and seed was collected.

For F_1 seed production susceptible parents (Table 4.2) were bud-pollinated with pollen from selected resistant individuals (denoted by * in Tables 3.3 and 3.4). Line R-o-18 (Table 4.2) was used as the susceptible parent in all of the *B. rapa*

Table 4.1 Turnip mosaic virus (TuMV) isolates used to test S_1, F_1, F_2 and B_1 generations of selected *Brassica rapa* and *B. napus* lines. √, isolate used to screen relevant generation, -, isolate not used.

Plant Line	Generation	TuMV Isolate				
		UK 1	UK 1M	CZE 1	CHN 5	CDN 1
B. napus						
22S	S_1	√	-	√	√	√
	F_1	√	-	√	√	√
	B_1	-	-	-	-	√
165	S_1	√	-	√	√	√
	F_1	√	-	√	√	√
	B_1	√	-	√	-	-
B. rapa						
Jong Bai No. 2	S_1	√	-	√	-	√
	F_1	√	√	√	-	√
	B_1	√	-	-	-	-
PI418957C	S_1	√	-	√	-	√
	F_1	√	-	√	√	√
	B_1	-	-	-	√	√
Jin G 55	S_1	√	-	-	√	√
	F_1	√	-	√	√	√
	B_1	-	-	-	-	√
BP058-4	S_1	√	-	√	-	√
	F_1	√	-	√	√	√
	B_1	-	-	-	√	√
Jong Bai No. 1	S_1	√	-	√	√	√
	F_1	√	-	√	√	√
	F_2	-	-	√	-	-
0-2	F_1	√	-	-	√	√
PI419105	F_1	√	-	-	√	√
PI418959	F_1	√	-	√	-	√

Table 4.2 Information about plant lines used as susceptible parent in crosses, including their phenotypic interaction with four turnip mosaic virus (TuMV) isolates.

Species	Lines used	Attributes	References	TuMV isolate (pathotype)			
				UK 1 (1)	CZE 1 (3)	CHN 5 (3)	CDN 1 (4)
Brassica rapa ssp. *trilocularis*	R-o-18[1]	Inbred line Yellow seeded Self-compatible	J. Salava, A.G. Sharpe & D.J. Lydiate, unpublished	+[2]	+	+	+
Brassica napus	N-o-1[1]	Doubled haploid line Brown seeded Self-compatible	Sharpe *et al.* (1995) Walsh *et al.* (1999)	0[3]	$+_N$[4]	$+_N$	+
	N-o-9[1]	Doubled haploid line Brown seeded Self-compatible	Sharpe *et al.* (1995) Walsh *et al.* (1999)	+	+	+	+

Notes: [1]Seed obtained from DJ Lydiate, Agriculture & Agri-Food Canada, Saskatoon, Canada
[2]Susceptibility; chlorotic infection observed on inoculated and uninoculated leaves, virus detectable by ELISA
[3]Apparent immunity; no symptoms observed on inoculated or uninoculated leaves, virus undetectable by ELISA or back inoculation
[4]Susceptibility; necrotic infection observed on inoculated and uninoculated leaves, virus detectable by ELISA

crosses. Lines N-o-1 and N-o-9 (Table 4.2) were used as susceptible parents in crosses for *B. napus* lines 22S and 165 respectively. All of the susceptible parents were self-compatible, therefore, for a cross to succeed selfing had to be prevented before the flower opened. The unopened flower bud was opened with tweezers. The flower was then emasculated by removal of the six stamens (four long and two short) and the stigma was rubbed with the desired pollen using a pollen-covered stamen from an open flower of a male parent. Up to six buds were pollinated per inflorescence, the remaining buds were removed. Each pollinated flower bud was labelled with the details of the cross and the inflorescences were enclosed in cellophane "bread bags" to prevent cross-pollination. Once the seed-pods had ripened (approx. 8 weeks after the last bud had been pollinated) and dried they were harvested and the seed collected.

For backcross (B_1) seed production susceptible parents (Table 4.2) were bud-pollinated using pollen from an F_1 individual, as described in Figure 4.1. The susceptible parents were self-compatible so were emasculated then pollinated as described above, with pollen from the desired F_1 individual. Alternatively, an F_1 individual was bud-pollinated using pollen from a self of the original resistant parent (denoted by * in Tables 3.3 and 3.4) as described in Figure 4.2. The F_1 were emasculated as described above to prevent selfing. Each pollinated flower bud was labelled with the details of the cross and the inflorescences enclosed in cellophane "bread bags" to prevent cross-pollination. Once the seed-pods had ripened (approx. 8 weeks after the last bud had been pollinated) and dried they were harvested and the seed collected.

For F_2 seed production, inflorescences at the unopened bud stage on an F_1 plant from each line were enclosed in cellophane "bread bags" to prevent cross pollination. As buds opened plants were shaken to encourage transfer of pollen from stamen to stigma. Once seed-pods had ripened and dried they were harvested and the seed collected. Seed was then stored as an alternative to virus test if B_1 seed production failed or B_1 seed germination was poor.

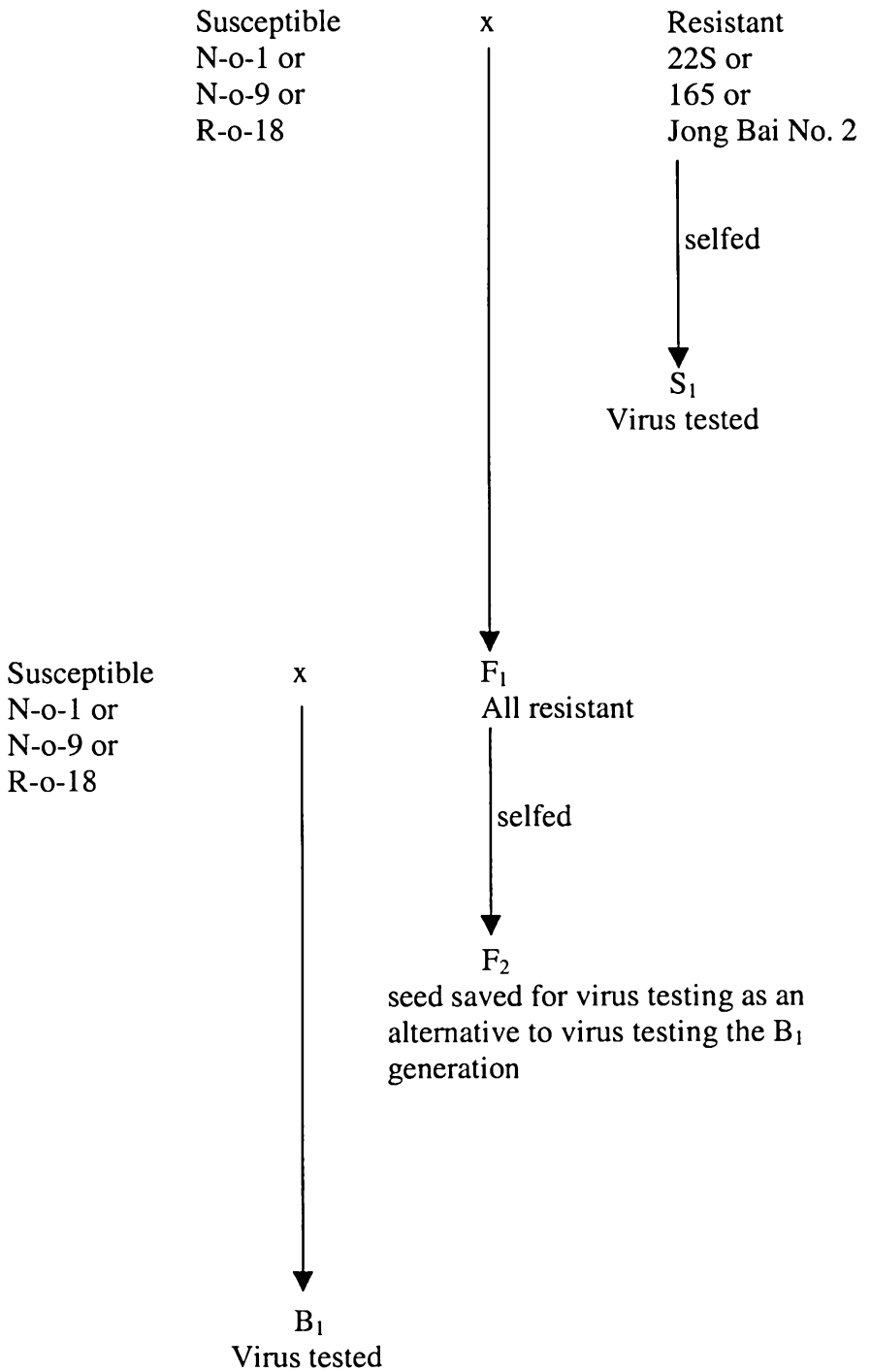


Figure 4.1 Development of populations for analysis of genetic basis of dominant resistance to turnip mosaic virus resistance in *B. napus* and *B. rapa*. N-o-1 was used as the susceptible parent in crosses with *B. napus* line 22S; N-o-9 was used as the susceptible parent in crosses with *B. napus* line 165; R-o-18 was used as the susceptible parent in crosses with *B. rapa* line Jong Bai No. 2.

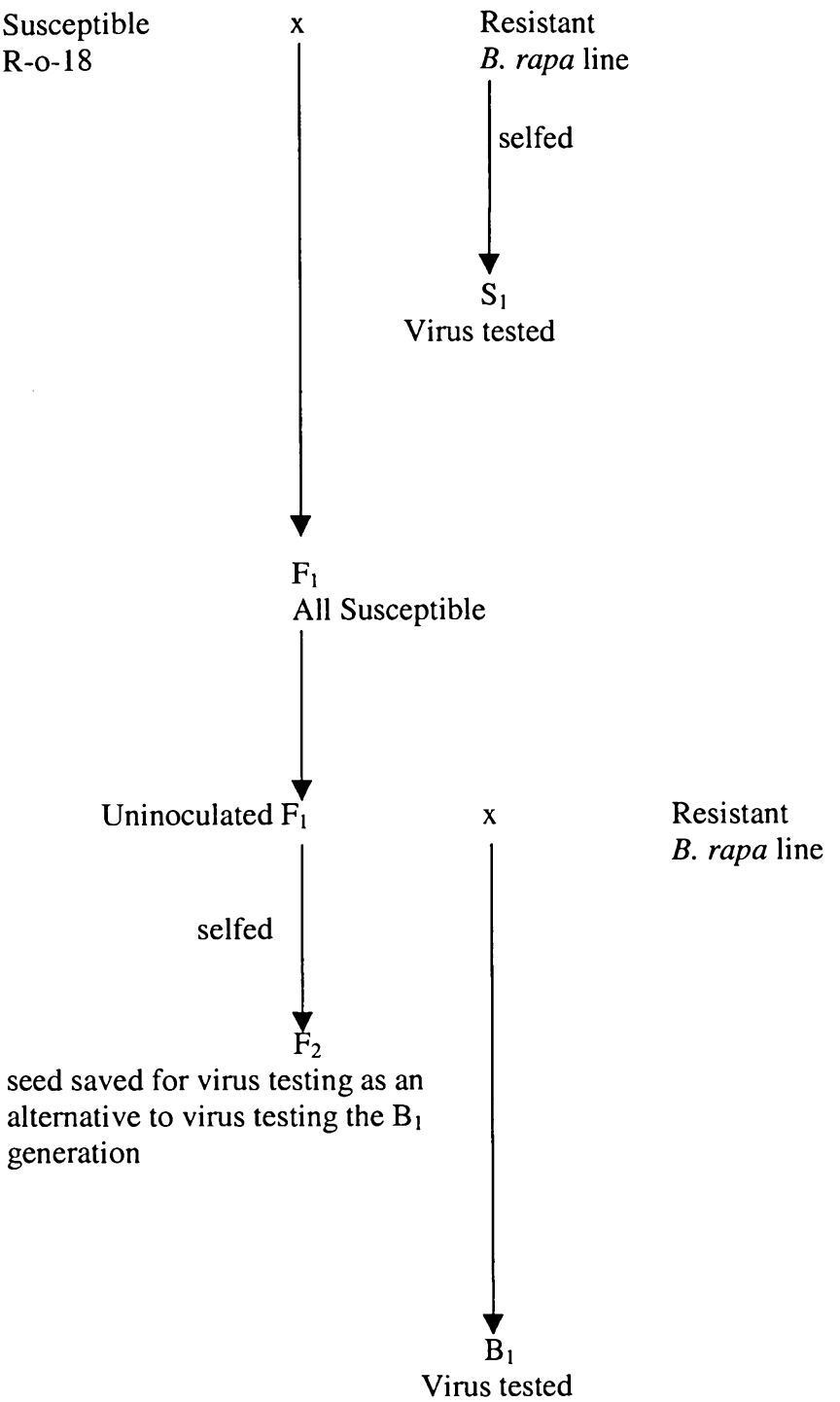


Figure 4.2 Development of populations for analysis of genetic basis of recessive resistance to turnip mosaic virus in *B. rapa*.

4.2.2 Virus Isolates

Virus isolates and the generation they were used to test are listed in Table 4.1. Virus isolates were maintained as described in section 3.2.4, Chapter 3. For a more detailed description of each isolate see Table 3.2, Chapter 3.

4.2.3 Plant Growth Conditions

Seeds from each generation were sown in 7cm pots and maintained in an insect-proof glasshouse at 18°C for the duration of the experiments.

4.2.4 Inoculation procedure

For each line between six and 27 F_1 individuals were inoculated, between six and 24 S_1 individuals were inoculated and between 40 and 113 B_1 individuals were tested with isolates listed in Table 4.1. For line Jong Bai No. 1, 118 F_2 individuals were tested with CZE 1 (Table 4.1) due to a lack of B_1 seed for this line. Number of individuals tested in each generation was dependent upon availability of seed. Plants were inoculated at the two-three true leaf stage using the inoculation procedure described in section 3.2.5, Chapter 3.

4.2.5 Assessment of Infection

Plants were assessed for infection as described in section 3.2.6, Chapter 3.

4.2.6 Statistical Analysis

Data of susceptible and resistant reactions were analysed by chi-square for goodness of fit to expected segregation ratios (genetic models of these segregation ratios are shown in the Appendix 1).

4.3 RESULTS

4.3.1 Selfed and F_1 progeny testing

The results from S_1 progeny testing are shown in Table 4.3. The S_1 generation for lines 0-2, PI418959 and PI419105 were not tested because amounts of seed produced through bud pollination were too low, even after treatment with 0.5% NaCl, to allow virus testing. The results from F_1 progeny testing of all lines are shown in Table 4.4.

If the F_1 plants were susceptible, as for crosses involving lines 0-2, PI419105, PI418959, PI418957C, Jin G 55, BP058-4 and Jong Bai No. 1 to all isolates tested and Jong Bai No. 2 with CZE 1 and CDN 1 (Table 4.4), it indicated that TuMV resistance was a recessive trait. TuMV infection causes decreased seed yield so in these instances an uninoculated F_1 individual was used as the maternal parent with an S_1 plant, derived from the resistant parent, as the pollen donor to produce a backcross (B_1) generation for each line. B_1 seed was not produced for *B. rapa* lines 0-2, PI419105 and PI418959 because they were too self-incompatible to produce sufficient amounts of seed for virus testing.

The identification of resistant F_1 plants, as for lines 22S, 165 and Jong Bai No. 2 (Table 4.4), indicated that TuMV resistance was a dominant trait. The resistance to TuMV in *B. rapa* line Jong Bai No. 2 is similar to that in *B. napus* conferred by the dominant allele, *TuRB01* (Walsh *et al.*, 1999) and in *B. rapa* another dominant allele, *TuRB01b* (Rusholme, 2000) in that it was effective against UK 1. A mutant of UK 1, UK 1M (Lehmann *et al.*, 1997), overcomes both *TuRB01* (Walsh *et al.*, 1999) and *TuRB01b* (Rusholme, 2000), causing a systemic necrotic phenotype. Consequently, Jong Bai No. 2 F_1 individuals were tested with UK 1M before continuing with the genetic analysis of the resistance in this line. Approximately half the F_1 individuals had resistance to UK 1M, the other half were susceptible. All Jong Bai No. 2 F_1 UK 1M susceptible plants were stunted

Table 4.3 Interaction of *Brassica rapa* and *B. napus* selfed (S_1) generations with different turnip mosaic virus (TuMV) isolates.

Plant Line	Virus isolate (pathotype)			
	UK 1 (1)	CZE 1 (3)	CHN 5 (3)	CDN 1 (4)
B. napus				
22S	10/10 $+_N$	10/10 $+_N$	10/10 $+_N$	24/24 0
165	10/10 0	10/10 0	10/10 0	10/10 +
B. rapa				
Jong Bai No. 2	6/6 0	6/6 0	-	4/6 0 2/6 R
PI418957C	1/3 0 2/3 +	2/2 0	-	2/2 0
Jin G 55	5/6 0 1/6 R_N	-	4/6 0 2/6 R	6/6 R
BP058-4	6/6 0	6/6 0	-	6/6 R
Jong Bai No. 1	4/6 0 1/6 R 1/6 R_N	6/6 0	-	4/6 R 2/6 +

Notes:
- 0 — Apparent immunity; no symptoms observed on inoculated or uninoculated leaves, virus not detectable by ELISA or back inoculation
- R — Resistance; chlorotic symptoms observed in inoculated leaves, virus detectable by ELISA; no symptoms observed in uninoculated leaves and no virus detectable by ELISA or back inoculation
- R_N — Resistance; necrotic symptoms observed in inoculated leaves, virus detectable by ELISA; no symptoms observed in uninoculated leaves and no virus detectable by ELISA or back inoculation
- \+ — Susceptibility; chlorotic infection observed in inoculated and uninoculated leaves, virus detectable by ELISA
- $+_N$ — Susceptibility; necrotic infection observed on inoculated and uninoculated leaves, virus detectable by ELISA
- \- — Line not tested with isolate

Table 4.4 Interaction of *Brassica rapa* and *B. napus* F_1 generations with different turnip mosaic virus (TuMV) isolates.

Plant Line	Virus isolate (pathotype)			
	UK 1 (1)	CZE 1 (3)	CHN 5 (3)	CDN 1 (4)
B. napus				
22S	10/10 0	10/10 $+_N$	10/10 $+_N$	20/20 0
165	10/10 0	10/10 0	10/10 0	20/20 +
B. rapa				
Jong Bai No. 2	9/27 0 15/27 + 3/27 $+_N$	14/16 + 2/16 $+_N$	-	16/16 +
PI418957C	14/25 + 11/25 $+_N$	6/6 +	10/10 +	15/15 +
Jin G 55	7/12 + 5/12 $+_N$	10/10 +	3/3 +	13/13 +
BP058-4	15/15 +	10/10 +	6/6 +	15/15 +
Jong Bai No. 1	13/16 + 3/16 $+_N$	6/6 +	10/10 +	16/16 +
0-2	6/6 $+_N$	-	6/6 +	6/6 +
PI419105	10/10 +	-	10/10 +	10/10 +
PI418959	4/10 + 6/10 $+_N$	9/9 +	-	10/10 +

Notes:
- 0 Apparent immunity; no symptoms observed on inoculated or uninoculated leaves, virus undetectable by ELISA or back inoculation
- \+ Susceptibility; chlorotic infection observed on inoculated and uninoculated leaves, virus detectable by ELISA
- $+_N$ Susceptibility; necrotic infection observed on inoculated and uninoculated leaves, virus detectable by ELISA
- \- Plants not tested with isolate

and had the mosaic phenotype (see Figure 4.3). Line Jong Bai No. 2 was taken forward for further characterisation because it had resistance to UK 1M and the susceptible plants were systemic mosaic which implied that different gene(s) were present in Jong Bai No. 2 to those identified by Walsh *et al.* (1999) and Rusholme (2000). For lines 22S, 165 and Jong Bai No. 2 a resistant F_1 individual was selected which was used to pollinate the susceptible parent, N-o-1, N-o-9 and R-o-18 respectively.

4.3.2 Results of virus testing B_1 generations

Virus testing of B_1 families indicated that the genetic basis of TuMV resistance could be grouped into three types: resistance determined by monogenic dominant alleles, monogenic recessive alleles and multigenic recessive alleles.

Genetic models for monogenic, dominant alleles that confer resistance to TuMV

The observed segregation data from virus testing B_1 generations of lines 22S and 165 fitted the expected segregation for a Mendelian model based on the action of a single dominant allele (Table 4.5). This model is appropriate if the original resistant individual was either homozygous for the dominant allele (genotype RR) or heterozygous (genotype Rr). The data from virus testing the F_1 (Table 4.4) and S_1 (Table 4.3) generations suggest that the original resistant individual was homozygous for the dominant allele for both lines 22S and 165 since all individuals were resistant. If a resistant F_1 individual (genotype Rr) was backcrossed with a susceptible individual (genotype rr) the B_1 generation would be expected to segregate one resistant, genotype Aa, to one susceptible, genotype aa (Figure 4.4, also see Appendix 1, Figure A1 for genetic model). Virus testing of the B_1 generation for lines 22S with CDN 1 and 165 with isolates UK 1 and CZE 1 produced observed segregation ratios that fit the proposed model (Table 4.5), see Figure 4.4. Figures 4.5 and 4.6 show typical phenotypes of 22S and 165 B_1 plants respectively.

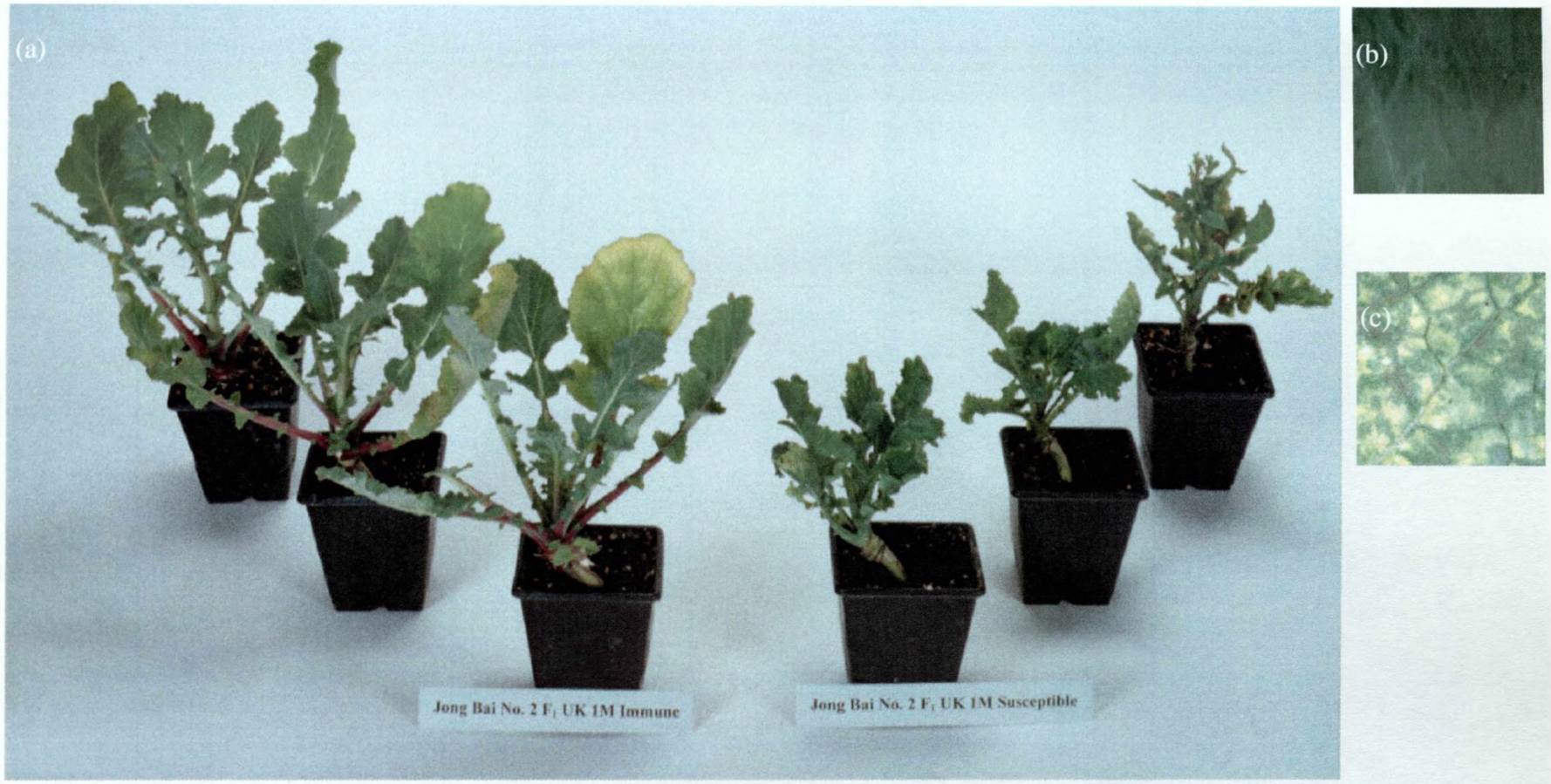


Figure 4.3 Jong Bai No. 2 F_1 individuals after challenge with turnip mosaic virus isolate UK 1M (a) Apparently immune plants are on the left hand side, susceptible plants are on the right hand side (b) Magnified healthy leaf from apparently immune plant (c) Magnified leaf from susceptible plant showing mosaic phenotype.

Table 4.5 Mendelian analysis of resistance to turnip mosaic virus isolates in first backcross (B_1) generations and F_2 generation of selected resistant lines of *B. rapa* and *B. napus*. χ^2-test values are presented for the fit of the observed resistant to susceptible ratio with expected ratios for the following genetic models: 1:1, 3:1, 1:3 and 10:54. χ^2-test and P values shown in bold are significant results.

				Resistant:Susceptible							
				Expected Segregation Ratio							
		Virus Isolate	Observed	1:1		3:1		1:3		10:54	
Plant Line	Generation	(Pathotype)	Segregation Ratio	χ^2	P	χ^2	P	χ^2	P	χ^2	P
22S	B_1	CDN 1 (4)	57:56	**0.009**	**0.920**	36.345	<0.001	39.012	<0.001	103.906	<0.001
165	B_1	UK 1 (1)	20:20	**0.000**	**1.000**	13.333	<0.001	13.333	<0.001	35.852	<0.001
	B_1	CZE 1 (3)	40:45	**0.294**	**0.590**	35.392	<0.001	22.059	<0.001	63.706	<0.001
Jong Bai No. 2	B_1	UK 1 (1)	31:27	**0.276**	**0.600**	14.368	<0.001	25.034	<0.001	62.938	<0.001
PI418957C	B_1	CHN 5 (3)	66:15	32.111	<0.001	**1.815**	**0.180**	137.815	<0.001	266.470	<0.001
	B_1	CDN 1 (4)	23:22	**0.022**	**0.880**	13.696	<0.001	16.363	<0.001	42.983	<0.001
Jin G 55	B_1	CDN 1 (4)	17:22	**0.641**	**0.420**	20.521	<0.001	7.188	0.007	23.134	<0.001
BP058-4	B_1	CHN 5 (3)	21:11	3.125	0.080	**1.500**	**0.220**	28.167	<0.001	60.681	<0.001
	B_1	CDN 1 (4)	23:65	20.045	<0.001	112.061	<0.001	**0.061**	**0.800**	69.794	<0.001
Jong Bai No. 1	F_2	CZE 1 (3)	15:103	65.627	<0.001	244.169	<0.001	9.503	0.002	**0.760**	**0.383**

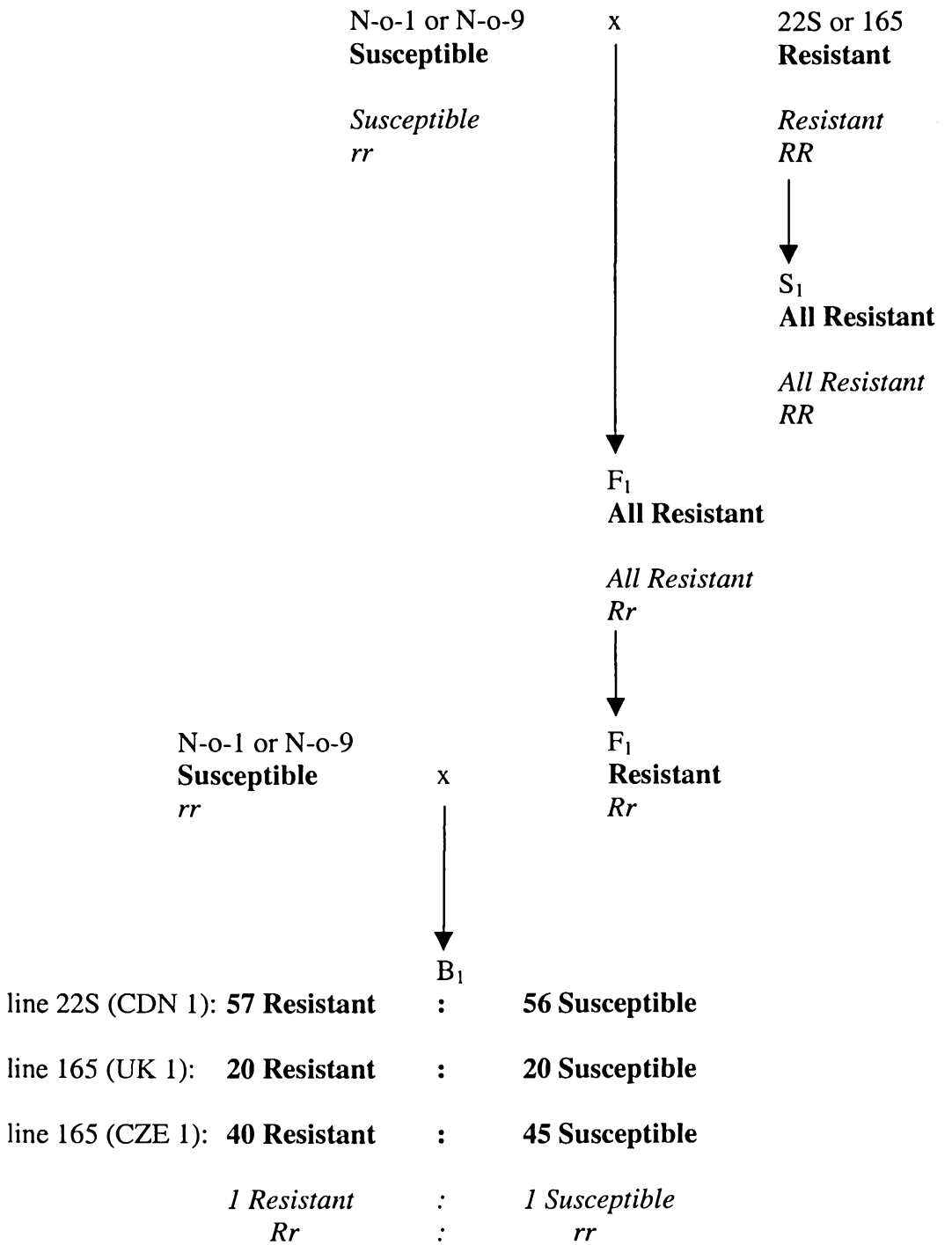


Figure 4.4 Genetic model based on the action of one dominant allele for resistance to turnip mosaic virus in line 22S to isolate CDN 1 and line 165 to isolates UK 1 and CZE 1. Observed phenotypes are shown in bold. Theoretical model phenotypes and genotypes are shown in italics.



Figure 4.5 Two individuals from *Brassica napus* line 22S backcross generation after inoculation with turnip mosaic virus (TuMV) isolate CDN 1. (a) Susceptible individual on left hand side, showing stunting and systemic mosaic; Resistant individual on right hand side, no TuMV replication could be detected. (b) Close up of leaf from susceptible plant showing mosaic phenotype. (c) Close up of symptomless leaf from resistant plant.

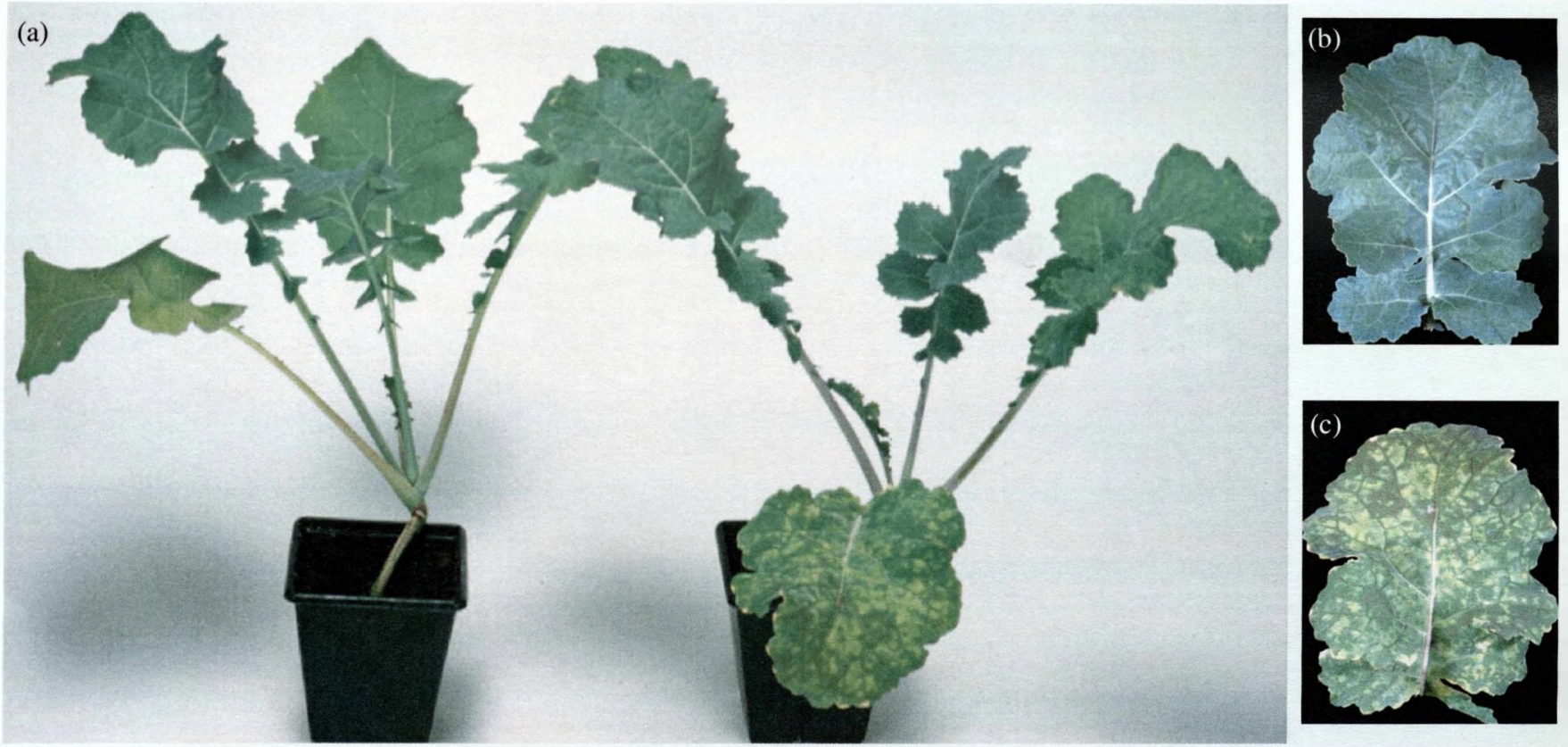


Figure 4.6 Two plants from *Brassica napus* line 165 after inoculation with turnip mosaic virus (TuMV) isolate CZE 1. (a) Resistant individual on left hand side, no virus could be detected; Susceptible individual on right hand side with systemic mosaic phenotype. (b) Close up of a symptomless leaf from the resistant individual. (c) Close up of leaf showing the mosaic phenotype from the susceptible individual.

For line Jong Bai No. 2, 58 B_1 individuals were tested with UK 1, 31 had resistance and 27 were susceptible (Table 4.5). The observed segregation data from virus testing Jong Bai No. 2 B_1 families with UK 1 fitted the expected segregation data for a Mendelian model based on the action of one dominant allele (χ^2=0.276, p=0.600, Table 4.5), see Figure 4.7 for genetic model. This model is appropriate if the original resistant Jong Bai No. 2 individual was either homozygous for the dominant allele (genotype RR) or heterozygous (genotype Rr). The data from virus testing the F_1 (Table 4.4) generation suggests that the original resistant individual was heterozygous for the dominant allele as there was segregation for resistance and susceptibility. If a resistant F_1 individual (genotype Rr) was backcrossed with R-o-18 (genotype rr), the B_1 generation would be expected to segregate one resistant (genotype Rr) to one susceptible (genotype rr) (Figure 4.7, also see Appendix 1, Figure A1 for genetic model), as observed (Table 4.5).

Genetic models for monogenic, recessive alleles that confer resistance to TuMV

The observed segregation data from testing B_1 generations of lines PI418957C and Jin G 55 with TuMV isolate CDN 1 fitted a Mendelian model based on the action of a single recessive allele (Table 4.5). This model is appropriate if the original resistant individual was homozygous for the recessive allele (genotype ss). Virus testing of the S_1 generation would have produced the resistant phenotype for all individuals (genotype ss), (Table 4.3) and the F_1 individuals would have been susceptible (genotype Ss), (Table 4.4), as observed. If an F_1 individual (genotype Ss) was backcrossed with a resistant individual (genotype ss) the B_1 generation produced would be expected to segregate one resistant (genotype ss) to one susceptible (genotype Ss), see Figure 4.8 and Appendix 1, Figure A2 for genetic model, as observed (Table 4.5).

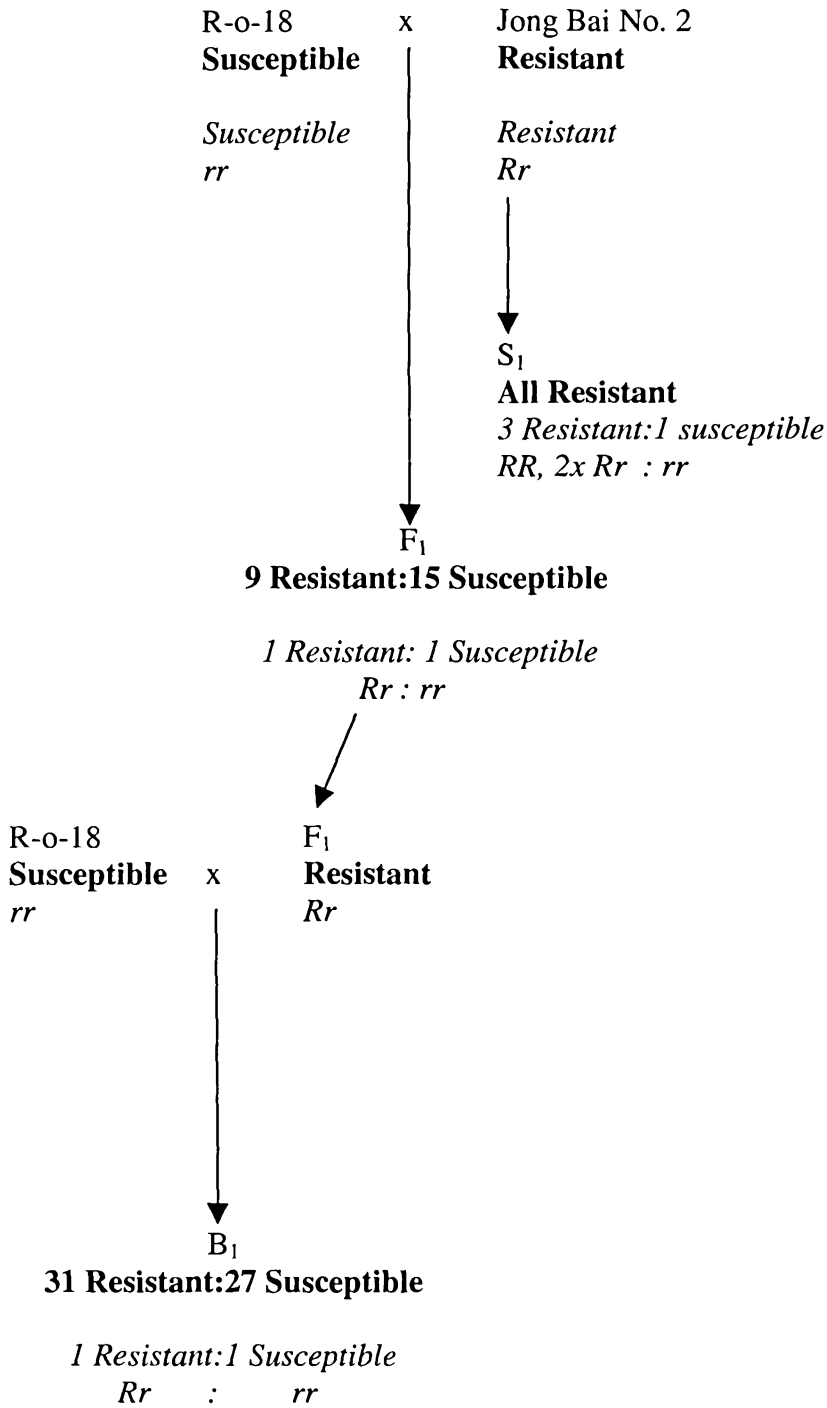


Figure 4.7 Genetic model based on the action of one dominant allele for resistance to turnip mosaic virus isolate UK 1 in line Jong Bai No. 2. Observed phenotypes are shown in bold. Theoretical phenotypes and genotypes are shown in italics.

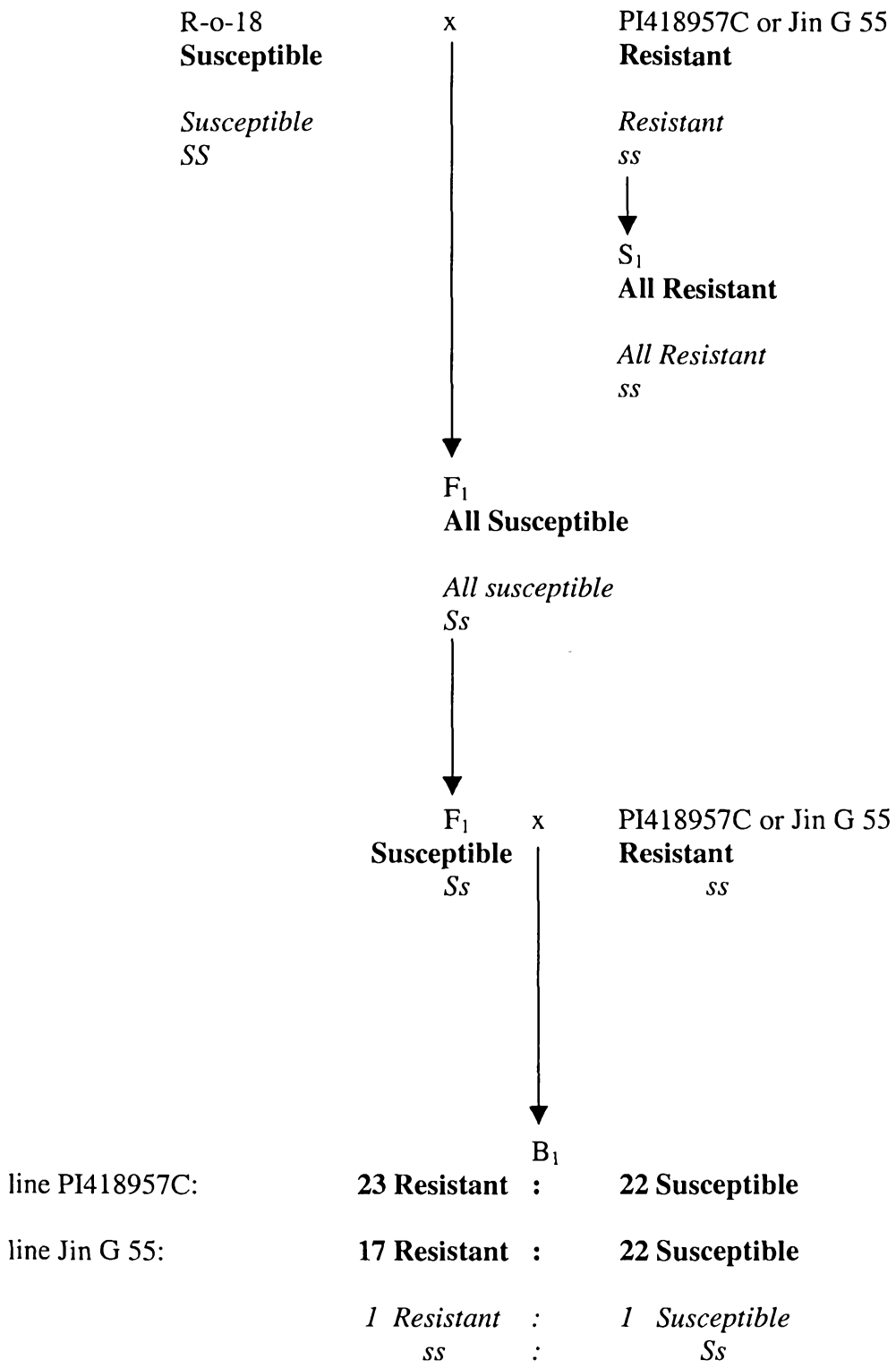


Figure 4.8 Genetic model based on the action of one recessive allele for resistance to turnip mosaic virus isolate CDN 1 in lines PI418957C and Jin G 55. Observed phenotypes are shown in bold. Theoretical phenotypes and genotypes are shown in italics.

Genetic models for multigenic, recessive alleles that control resistance to TuMV

The observed segregation data from testing B_1 generations of lines PI418957C and BP058-4 with TuMV isolate CHN 5 fitted a Mendelian model based on the action of recessive alleles at two epistatic unlinked loci, where homozygous alleles at a single loci are required at any one time (Table 4.5). This model is appropriate if the original resistant individual was homozygous for the recessive alleles at both loci (genotype aabb). Virus testing of the S_1 generation would have produced uniformly resistant phenotypes (genotype aabb), and the F_1 individuals would have all been susceptible (genotype AaBb), as observed (Table 4.4). If an F_1 individual (genotype AaBb) was backcrossed with a resistant individual (genotype aabb) a B_1 generation would be produced that was expected to segregate three resistant (genotypes aabb, aaBb, Aabb) to one susceptible (genotype AaBb), see Figure 4.9 and Appendix 1, Figure A3 for genetic model, as observed (Table 4.5).

The observed segregation data from testing the B_1 generation of line BP058-4 with TuMV isolate CDN 1 fitted a Mendelian model based on the action of recessive alleles at two complementary loci (Table 4.5). This model is appropriate if the original resistant individual was homozygous for the recessive alleles (genotype ccdd). Virus testing of the S_1 generation would have produced uniformly resistant phenotypes (genotype ccdd), (Table 4.3) and the F_1 individuals would have been susceptible (genotype CcDd), (Table 4.4). If an F_1 individual (genotype CcDd) was backcrossed with a resistant individual (genotype ccdd) the B_1 generation would be expected to segregate one resistant (genotype ccdd) to three susceptible (genotypes CcDd, ccDd, Ccdd) upon virus testing (see Figure 4.10 and Appendix 1, Figure A4 for genetic model), as observed (Table 4.5).

The observed segregation data from testing the F_2 generation of line Jong Bai No. 1 with TuMV isolate CZE 1 fitted a Mendelian model based on the action of recessive alleles at three loci where any two of the three loci were complementary and epistatic (Table 4.5). This model is appropriate if the original resistant

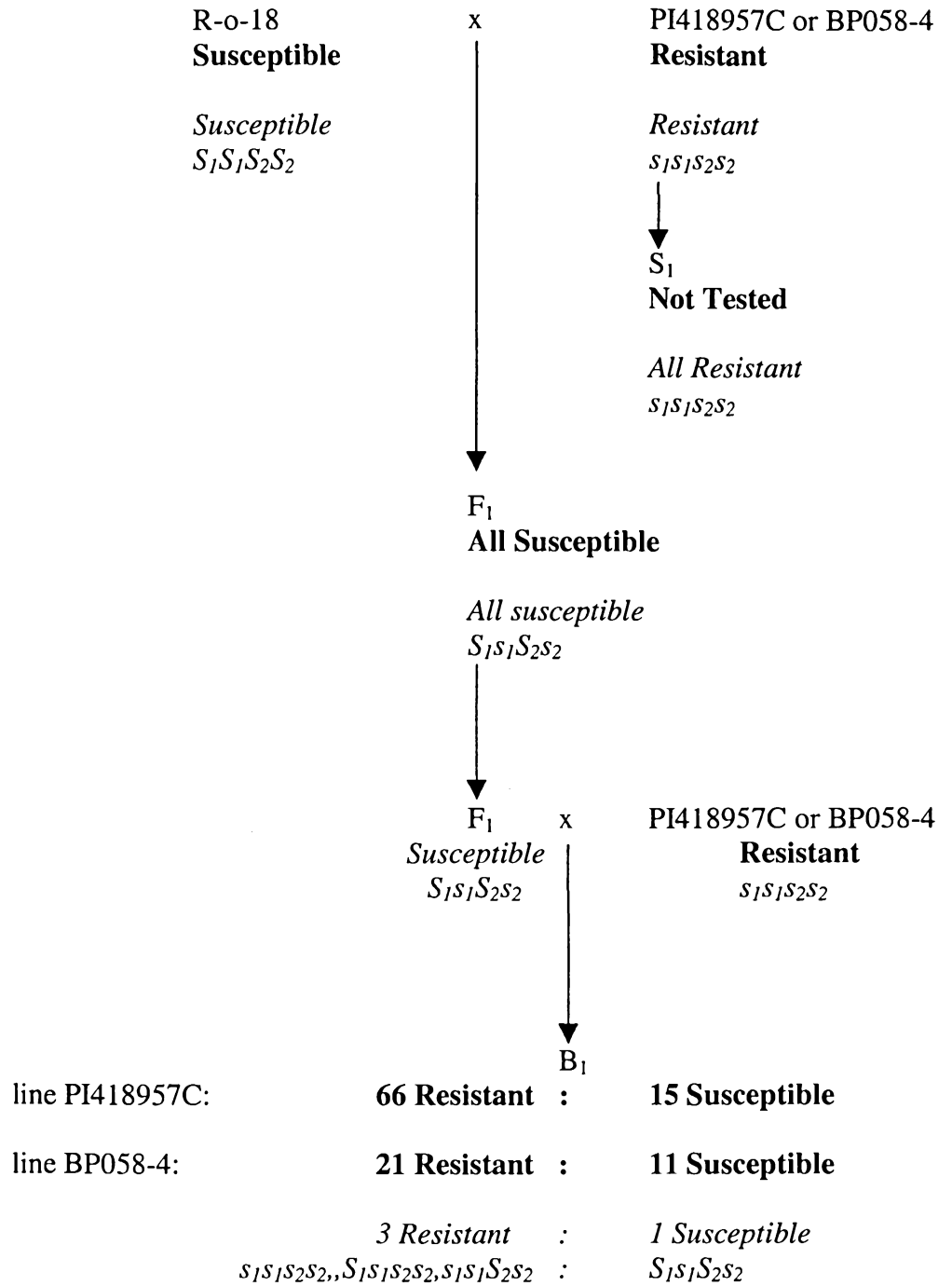


Figure 4.9 Genetic model based on the action of recessive alleles at two loci where homozygous alleles at either loci are required for resistance to turnip mosaic virus isolate CHN 5 in lines PI418957C and BP058-4. Observed phenotypes are shown in bold. Theoretical phenotypes and genotypes are shown in italics.

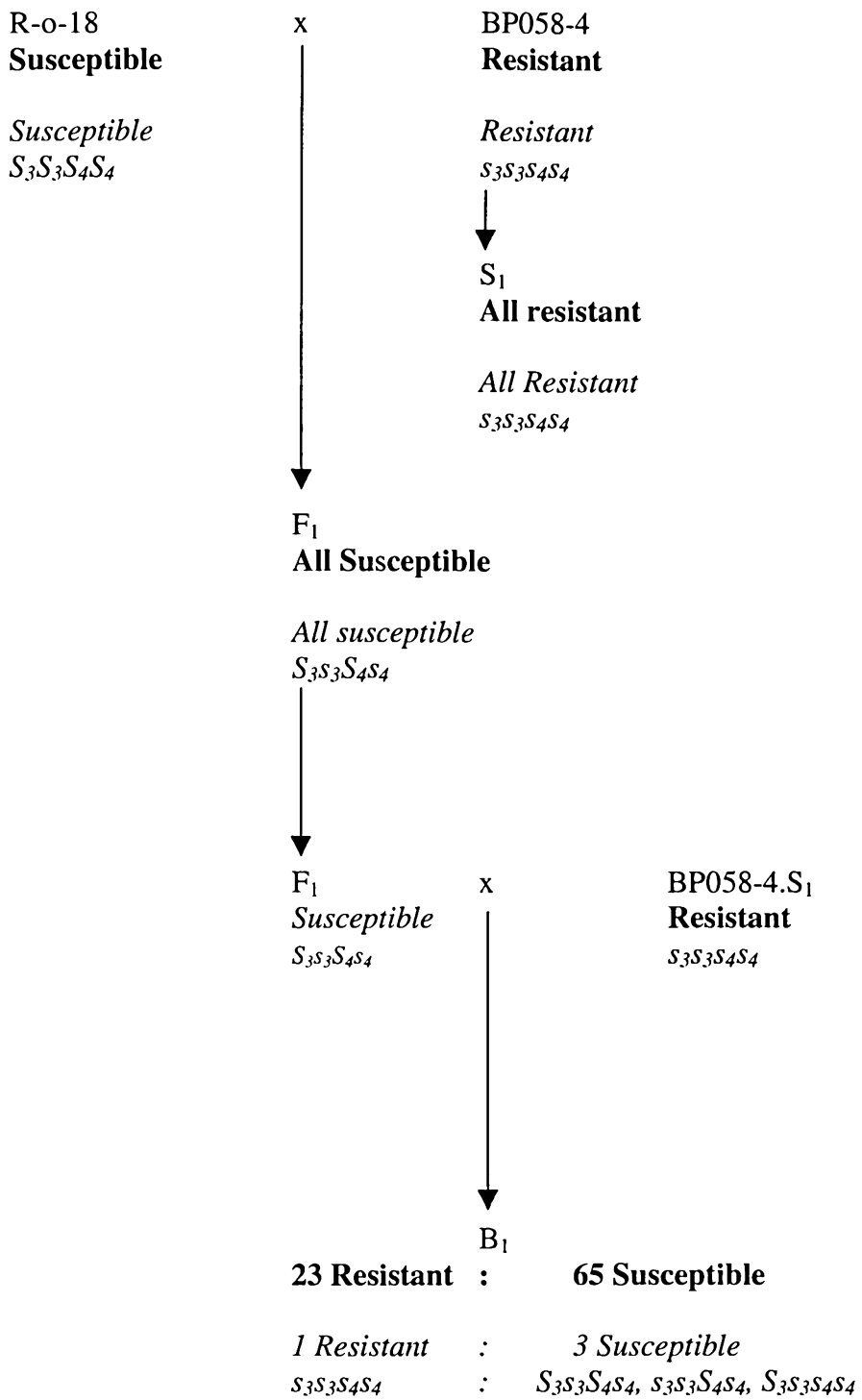
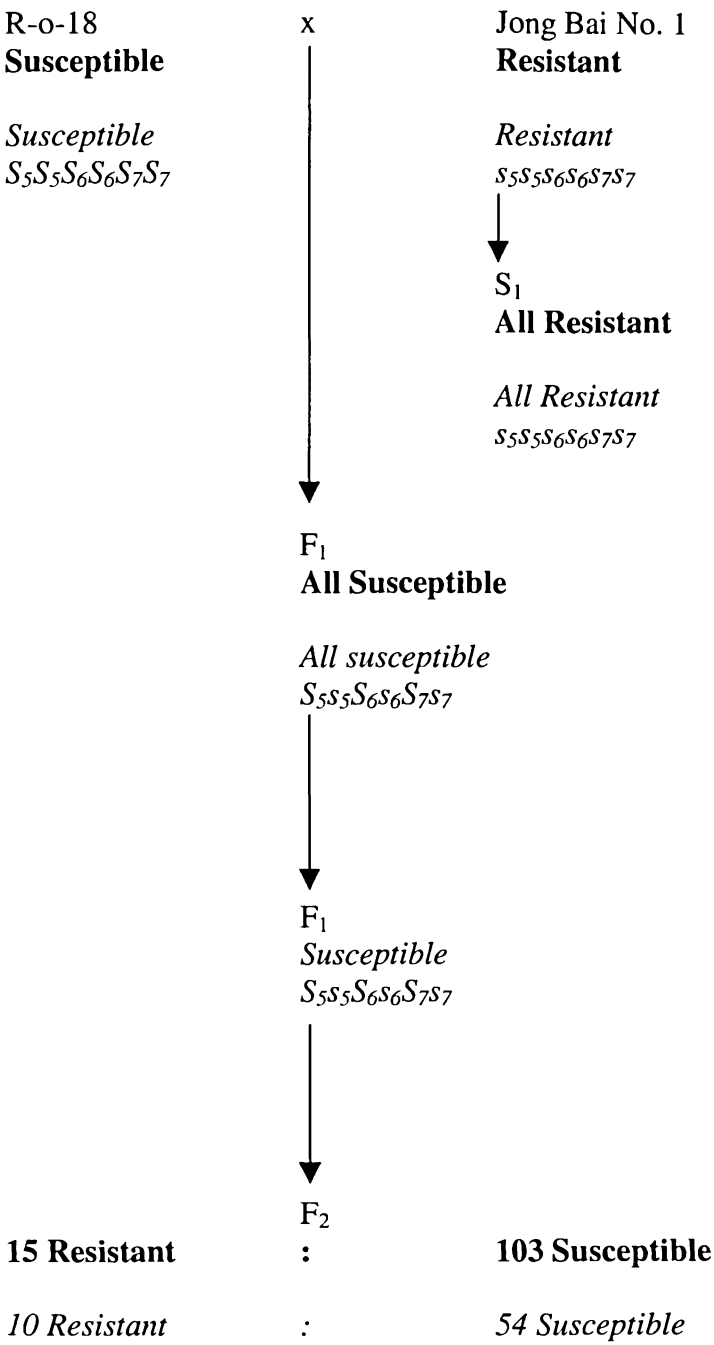


Figure 4.10 Genetic model based on the action of recessive alleles at two loci for resistance to TuMV isolate CDN 1 in *B. rapa* line BP058-4. Observed phenotypes are shown in bold. Theoretical phenotypes and genotypes are shown in italics.

individual was homozygous for the recessive alleles at all three loci (genotype eeffgg). Virus testing of the S_1 generation would produce all individuals with resistant phenotype (genotype eeffgg), as observed (Table 4.3) and all the F_1 individuals would be expected to be susceptible (genotype EeFfGg), as observed (Table 4.4). Virus testing of the subsequent F_2 generation would be expected to produce a segregation ratio of 10 resistant to 54 susceptible, see Figure 4.11 and Appendix 1, Figure A5 for genetic model, as observed (Table 4.5).



see appendix, figure 5 for genotypes of resistant and susceptible individuals

Figure 4.11 Genetic model based on the action of recessive alleles at three loci where homozygous alleles at two of the loci are required for resistance to turnip mosaic virus isolate CZE 1 in *B. rapa* line Jong Bai No. 1. Observed phenotypes are shown in bold. Theoretical phenotypes and genotypes are shown in italics.

4.4 DISCUSSION

The results from my study have extended our knowledge of the genetic basis of resistance to TuMV in *Brassica* spp. Table 4.6 summarises the types of resistance present to TuMV isolates in the different *Brassica* lines and the number of alleles required for resistance to be expressed.

I have confirmed that resistance to TuMV isolate UK 1 in *B. napus* line 165 is determined by a single dominant allele (Table, 4.6) (Walsh, 1989). The analysis of the genetic basis of resistance of line 165 to TuMV isolate CZE 1 has not been previously attempted, my results indicate that resistance is conferred by a single dominant allele (Table 4.6). Shattuck & Stobbs (1987) also described the presence of a single dominant allele in line 165 that conferred resistance to a Canadian TuMV isolate. In *B. napus* line 22S the resistance to CDN 1 also appeared to be conferred by a single dominant allele (Table 4.6). My results for these resistance sources in *B. napus* lines are similar to previous studies where only single dominant alleles (so called "major gene" or "R gene" resistance) conferring TuMV resistance in *B. napus* have been identified (Shattuck & Stobbs, 1987; Bradshaw *et al.*, 1989; Walsh, 1989; Walsh *et al.*, 1999).

Resistance to UK 1 in *B. rapa* line Jong Bai No. 2 also appears to be conferred by a single dominant allele. One other single dominant allele, *TuRB01b*, conferring resistance to UK 1 has been identified in *B. rapa* (Rusholme, 2000). However, the resistance identified by Rusholme (2000) is probably different to that identified in my study, because the 'resistances' have different specificities of interaction with other TuMV isolates. The resistance identified by Rusholme (2000) produces a systemic necrotic reaction upon inoculation with pathotype 3 isolates, including UK 1M, whereas Jong Bai No. 2 is apparently immune to UK 1M and produces both systemic mosaic and systemic necrosis reactions with other pathotype 3 isolates.

Table 4.6 Summary of number of alleles required for resistance/apparent immunity to relevant TuMV isolate and the mode of inheritance of alleles.

Plant Line	TuMV Isolate	TuMV Pathotype	Resistance Phenotype	No. alleles	Mode of inheritance
B. napus					
22S	CDN 1	4	0[1]	1	Dominant
165	UK 1	1	0	1	Dominant
	CZE 1	3	0	1	Dominant
B. rapa					
Jong Bai No. 2	UK 1	1	0	1	Dominant
PI418957C	CHN 5	3	R[2]	2, 1 required	Recessive
	CDN 1	4	R	1	Recessive
Jin G 55	CDN 1	4	R	1	Recessive
BP058-4	CHN 5	3	0	2, 1 required	Recessive
	CDN 1	4	R	2	Recessive
Jong Bai No. 1	CZE 1	3	R	3, 2 required	Recessive

Notes: [1]Apparent immunity; no symptoms observed on inoculated or uninoculated leaves, virus not detectable by ELISA or back inoculation
[2]Resistance; chlorotic symptoms observed in inoculated leaves, virus detectable by ELISA; no symptoms observed in inoculated leaves and no virus detectable by ELISA or back inoculation

The TuMV resistance identified in Jong Bai No. 2 is distinct from the resistance of other *B. rapa* lines characterised in this chapter because it is conferred by a single dominant allele. In contrast, TuMV resistance in the other *B. rapa* lines tested appear to be determined by one or more recessive alleles. Furthermore, the results of inoculation of B_1 families of these *B. rapa* lines with recessive TuMV resistance indicated that the resistance was under more complex genetic control in some lines. Lines PI418957C and BP058-4 appeared to require homozygosity for the "resistant" allele at one of two loci to provide resistance to CHN 5, whereas recessive resistance to CDN 1 was determined by only a single locus in line PI418957C. In line BP058-4 resistance to CDN 1 was controlled by two complementary loci.

Several examples of recessive alleles conferring resistance to potyviruses have been reported (Drijfhout, 1978; Schaad & Carrington, 1996; Nicolas *et al.*, 1997; Murphy *et al.*, 1998; Hamalainen *et al.*, 2000). The complex nature of resistances to potyviruses are unusual in that the majority of characterised plant resistance alleles to other viruses that have been identified so far are under simple genetic control (Fraser, 1992). Multigenic control of resistance to bean common mosaic virus (BCMV) in *Phaseolus vulgaris* is one of the most characterised systems of resistance to a potyvirus. In this system at least five strain-specific resistance alleles, *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2} and *bc-3*, (Drijfhout, 1978) require an additional recessive allele, *bc-u* which does not appear to be strain specific, for expression of resistance to BCMV (Drijfhout, 1978; Kelly *et al.*, 1995). The *bc* system has been expanded by Donovan (2000).

Another example of multigenic resistance against potyviruses is in pepper where several genetic factors are involved in resistance to Potato Virus Y (PVY), pathotype 0 (Caranta & Palloix, 1996). The same genetic factors involved in resistance to PVY-0 are also involved in resistance to 'potyvirus E' and some are also required, along with additional genes, for resistance to PVY pathotypes 1 and 2 (Caranta & Palloix, 1996). The TuMV resistance in *B. rapa* lines that I have identified could involve similar multigenic systems to these examples. For

example, BP058-4 appeared to require one of two recessive alleles to provide resistance to CHN 5. The χ^2-value for this model (Table 4.5) suggests that the true genetic model may not be this simple. Previous work on the genetics of resistance to TuMV in line BP058 by Suh *et al.* (1996) also supports a more complex resistance model. Likewise, the broad-spectrum resistance to TuMV in another *B. rapa* line, 0-2, appears to be under multigenic control (Yoon *et al.*, 1993; Suh *et al.*, 1995; Suh *et al.*, 1996). Multigenic resistance systems often occur when resistance is dependant upon either the cumulative effect of several gene products or the efficiency of major resistance gene products is dependent upon the activity of signal transduction pathway gene products (Fraser, 1990, 1992).

Although single gene mediated resistance to some viruses has been durable, e.g. resistance conferred by the *N* gene, multigenic systems are more complex to understand and exploit than simple monogenic systems. Theoretically, the multigenic systems may represent a more durable form of resistance, forcing the virus to achieve multiple mutations to overcome them. It is possible that such multigenic resistance systems result from the evolution of complex gene-for-gene relationships between host and virus.

The most effective method of deploying virus resistance is likely to be pyramiding of resistance genes through multilines or mixtures, to prevent the "boom-bust" cycle re-occurring (Crute & Pink, 1996; Pink & Puddephat, 1999). Before this can be achieved more information must be obtained on the underlying genetics involved in the resistance sources identified in this study. The next step in achieving this is to identify molecular markers linked to TuMV resistance in order to position the genes on genetic maps. The identification of markers and the subsequent mapping of the genes will aid in confirming or disproving the hypothesised genetic models proposed in this chapter (see Chapter 5). Also, any generated marker information can be combined with further genetic analyses of host resistance, to provide a tool for marker-assisted-selection breeding programmes in the future.

CHAPTER 5

MOLECULAR ANALYSIS OF TuMV RESISTANCE IN *BRASSICA RAPA & B. NAPUS*

5.1 INTRODUCTION

Molecular markers can be used to assist in the deployment of alleles conferring resistance to disease through marker assisted selection (MAS) in breeding programmes (Han *et al.*, 1997; Young, 1999). They can also be used to position resistance genes on genetic linkage maps (Walsh *et al.*, 1999; Rusholme, 2000), which facilitates map-based cloning (Martin *et al.*, 1993; Mindrinos *et al.*, 1994; Salmeron *et al.*, 1994; Ori *et al.*, 1997). Once the genes have been isolated they can be rapidly deployed via genetic transformation to produce transgenic crops resistant to disease (Pink & Puddephat, 1999). As previously discussed (see Chapter 1) there are several types of molecular markers, e.g. random fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites.

The AFLP technique has several advantages over the RAPD and RFLP techniques. AFLP results are more reproducible than those generated by RAPD because of the stringent reaction conditions applied in the AFLP technique (Joshi *et al.*, 1999). The technique also has several advantages over RFLP because it is less time consuming and, if fluorescent labels are used, it is less hazardous and less expensive. The main advantage AFLPs have over RFLP is that they have the potential to detect large numbers of polymorphic loci (Joshi *et al.*, 1999). Microsatellites also have advantages over RAPD and RFLP techniques because they are co-dominant markers, detect multiple alleles and are highly reproducible. AFLP and microsatellite techniques have a further advantage through the development of fluorescent systems which allow several primer pairs to be combined in a single amplification reaction, which reduces the time in which a large volume of results can be generated.

In order to further characterise the resistances to TuMV identified in lines of *B. rapa* and *B. napus* (Chapter 3) molecular markers linked to allele(s) controlling resistance are required. Lines 22S, 165 and Jong Bai No. 2 were selected for molecular analysis because genetic characterisation suggested that, in these lines, single dominant alleles conferred TuMV resistance (Chapter 4). The principle aim of this work was to identify markers linked to alleles conferring TuMV resistance and to use these linked markers to position the alleles on a *B. napus* linkage map (Sharpe *et al.*, 1995).

Michelmore *et al.* (1991) have described the identification of linked markers to a resistance gene by bulk segregant analysis (B-SA) using RAPD. B-SA is when the DNA from resistant individuals is pooled to form a resistant bulk and the DNA from susceptible individuals is pooled to form a susceptible bulk. The bulks are then used to identify markers linked to resistance, rather than using each individual, hence B-SA speeds up the generation of results. It also reduces the probability of detecting unlinked markers (Michelmore *et al.*, 1991). Here, B-SA has been used to identify AFLP markers linked to TuMV resistance. These linked markers were then used to anchor the resistance to a linkage group on a *B. napus* genetic linkage map (Sharpe *et al.*, 1995). Microsatellite markers have also been established on the *B. napus* genetic linkage map and can be used to determine mapping intervals (D. Lydiate & A. Sharpe, personal communication). Microsatellites have been deployed through B-SA to anchor the TuMV resistance to a linkage group where no linked AFLP markers were identified. They were also used to estimate map intervals.

Five TuMV resistance genes have already been mapped in *B. rapa* and *B. napus* (Walsh *et al.*, 1999; Rusholme, 2000). Four of these map to the A-genome and one maps to the C-genome. Two of these TuMV resistance genes, *TuRB01* and *TuRB01b*, have been mapped to the same location on linkage group N6. Experimental evidence suggests that evolution of gene clusters for sources of resistance to diseases occurs (Hammond-Kosack & Jones, 1997; Geffroy *et al.*, 1999; Richter & Ronald, 2000; Holub 2001) and this may also be true for TuMV

resistance genes. Mapping the resistances in lines 22S, 165 and Jong Bai No. 2 will provide further insights for evolution of gene clusters for TuMV resistance.

5.2 MATERIALS & METHODS

5.2.1 Source Material

Plants from three populations derived from 22S, 165 and Jong Bai No. 2 were used. For identification of markers linked to alleles conferring resistance to TuMV, leaves were collected from the original resistant individual, susceptible parent and subsequent F_1 for each population, (Chapter 4). For the 22S and Jong Bai No. 2 B_1 populations, leaves were collected from 20 TuMV resistant individuals and 20 TuMV susceptible individuals (Chapter 4). For the 165 B_1 population leaves were collected from ten UK 1 resistant individuals, ten UK 1 susceptible individuals, 20 CZE 1 resistant individuals and 20 susceptible individuals (Chapter 4). Each leaf sample from an individual plant was placed in a bread bag, immediately frozen in liquid nitrogen and freeze-dried for 3 days to ensure leaf material was dry. Once leaf material was dry it was stored at -20°C in a plastic bag, containing self-indicating silica gel, until required.

5.2.2 DNA Extraction

The Phytopure Method

Genomic DNA was extracted from individuals from lines 22S and Jong Bai No. 2 using the Phytopure Kit according to the manufacturers' instructions (Nucleon Biosciences).

For each line, 20mg freeze-dried leaf material from each of the resistant individuals, F_1, B_1 individuals and susceptible parents, N-o-1 for line 22S and R-o-18 for line Jong Bai No. 2 (Chapter 4), were ground to a fine powder using a sterile mortar and pestle. The powder was transferred to a 1.5ml microfuge tube and 600µl "Nucleon Biosciences Reagent 1" was added. Once the powder had dissolved, to remove any traces of RNA, RNase was added to a final

concentration of 20μg.ml^{-1} and the mixture was incubated at 37°C for 30 minutes. After incubation, 200μl "Nucleon Biosciences Reagent 2" was added and the tube inverted several times until a homogeneous mixture was obtained. The mixture was then incubated in a water bath at 65°C for 10 minutes with constant agitation. After incubation the sample was placed on ice for 20 minutes. Then 500μl ice cold chloroform and 100μl "Nucleon Phytopure DNA extraction resin suspension" were added to the sample and the mixture was shaken at room temperature for 10 minutes. The sample was centrifuged at 1300x *g* for 10 minutes and the upper phase was transferred to a fresh 1.5ml microfuge tube. An equal volume of ice cold propan-2-ol was added to the sample and the tube gently inverted until the DNA precipitated. The sample was then centrifuged at 4000x *g* for 5 minutes, the supernatant discarded and the DNA pellet washed with 70% ethanol. The sample was centrifuged at 4000x *g* for 5 minutes to repellet the DNA, the supernatant discarded and the pellet air-dried for 10 minutes. The pellet was resuspended in 300μl sterile distilled water (SDW).

The Kirby Mix Method

Genomic DNA was extracted from line 165 material using the Kirby Mix method as described by Sharpe *et al.* (1995).

0.5g freeze-dried leaf material from each of resistant individual (165), susceptible individual (N-o-9), F_1 and B_1 individuals, was milled for 20 minutes until a fine powder was formed. The powder was transferred to a 50ml polypropylene tube and 15ml Kirby mix added (10g.l^{-1}, sodium tri-isopropylnapthalene sulphonate; 72.5g.l^{-1}, 4-aminosalicylic acid; 25ml.l^{-1}, 2M tris-HCl pH-8; 60ml.l^{-1}, buffer saturated phenol; 900ml.l^{-1}, deionized water; all measured and mixed together in a foil covered bottle in a fume hood). The sample was vortexed for 10-15 seconds and then mixed on a shaking platform for 30 minutes. 10ml of phenol/chloroform was added, the sample was vortexed for 10-15 seconds and mixed on a shaking platform for 5 minutes. The sample was then centrifuged at 3000rpm for 20 minutes and then the upper phase transferred to a fresh 50ml polypropylene tube

containing 1.5ml 3M sodium acetate, pH 6.0 and mixed gently. 9ml of propan-2-ol was added to the mixture, the sample mixed gently and then left in the dark at room temperature overnight. The sample was then centrifuged at 3000rpm for 20 minutes, the supernatant discarded and the pellet air-dried for 30 minutes. The pellet was then resuspended in 2ml 1 x TE (10mM tris-HCl, 1mM EDTA, pH8.0) with 40μg.ml^{-1} RNase and the sample incubated at 37°C until the pellet was totally resuspended. The resuspended pellet was transferred to a 15ml polypropylene tube, 2ml phenol/chloroform added, the sample vortexed and mixed on a shaking platform for 5 minutes. The sample was then centrifuged at 3000rpm for 20 minutes and the upper phase transferred to a fresh 15ml polypropylene tube containing 180μl of 3M sodium acetate, pH6.0. The sample was gently mixed, then 1.2ml of propan-2-ol added. The sample was mixed gently and left in the dark at room temperature overnight. The sample was then centrifuged at 3000rpm for 20 minutes, the supernatant poured off and the pellet air-dried. The dry pellet was then resuspended in 0.25ml 1 x TE by incubating at 37°C until the pellet was totally dissolved.

5.2.3 DNA Quantification

1x TBE Agarose Gel Electrophoresis

The amount of genomic DNA obtained using the Phytopure Kit (Nucleon Biosciences) was quantified by agarose gel electrophoresis.

Agarose was added to a final concentration of 1.5g.l^{-1} in 1 x TBE (89mM Tris, 89mM boric acid, 2mM EDTA pH 8.0). The agarose was dissolved by heating in a microwave oven, then was cooled until "hand-hot." Ethidium bromide was then added to a concentration of 0.5μg.ml^{-1}. The agarose was poured into a gel tray containing combs and left to set. Once the gel was set, it was placed in a horizontal gel tank and immersed in 1x TBE buffer and the combs were removed to form wells. Each DNA sample was diluted in 6x loading buffer (60% glycerol, 0.2% bromophenol blue, 0.2% xylene cyanol FF, 60mM EDTA), with 10μl of

undigested genomic DNA diluted in 10µl loading buffer. The molecular weight marker used was Hyperladder (Bioline). Gels were run at 100V for one hour, to prevent distortion of the gel, and the genomic DNA was visualised using a UV transilluminator. Sample DNA was quantified by comparison with DNA of known concentration and molecular weight from Hyperladder.

Fluorometric analysis and 1x TAE Agarose Gel Electrophoresis

The amount of genomic DNA obtained using the Kirby mix method was quantified using a fluorometric technique and by agarose gel electrophoresis.

The genomic DNA was digested to give a stable fluorometric reading. The restriction digest was carried out in a final volume of 30µl, with 10µl genomic DNA prep, 3µl "H-Buffer", 3µl 40mM spermidine, 0.6µl *Eco*RI ($40u.\mu l^{-1}$), 13.4µl SDW. Reactions were incubated overnight at 37°C. After the overnight incubation the fluorometer excitation wavelength was set at 365nm and the emission wavelength was set at 458nm. While the fluorometer was warming up a $1.0\mu g.ml^{-1}$ solution of Hoechst 33258 (Sigma B-2883) was made by adding 5µl of the $2mg.ml^{-1}$ stock to 10ml of 10 X TEN buffer (100mM Tris pH7.4, 10mM EDTA, 500mM NaCl, autoclaved and stored at room temperature). 3µl of standard known concentration DNA samples were aliquoted into 280µl $1.0\mu g.ml^{-1}$ Hoechst solution. Fluorometric readings of DNA of known concentrations were then taken. A standard curve of known DNA concentrations against fluorometric readings was plotted. Readings of sample DNA were done in the same way as those of the standard DNA. The concentration of sample DNA was estimated from the standard curve of DNA of known concentrations.

2-4µg of the remaining digested DNA sample was viewed on a 1x TAE 0.8% agarose gel to check the quality of the DNA. Agarose was added to a final concentration of $8g.l^{-1}$ in 1x TAE (40mM Tris, 40mM glacial acetic acid, 1mM EDTA, pH8.0). The preparation of 1x TAE gels was the same as for 1x TBE gels.

The 1x TAE gels were electrophoresed at 25V overnight and the DNA was visualised by placing the gel on a UV transilluminator.

5.2.4 AFLP Analysis

As described in section 1.5.2, Chapter 1 the AFLP procedure involves the restriction digestion of genomic DNA with two enzymes, in this case the enzymes were *Eco*RI and *Mse*I. Before beginning the AFLP procedure the genomic DNA was digested with *Mse*I and the product run out on a 1.5% 1x TBE gel as described in section 5.2.3.

The AFLP Small Plant Mapping Kit was used for the AFLP procedure according to the manufacturers instruction (AFLP Plant Mapping Protocol, PE Applied Biosystems).

Samples were run out on polyacrylamide gels on PE Biosystems ABI 377 using standard conditions, including an internal lane standard (GeneScan Reference Guide, 1997). GeneScan 3.1 was used to analyse the AFLP data.

5.2.5 Microsatellite Analysis

Fluorescently-labelled primers for *B. napus* microsatellites were obtained from A.G. Sharpe and D.J. Lydiate, AAFC, Saskatoon (The primers were labelled with one of three dyes, HEX, 6-FAM or TET, which fluoresce at distinct wavelengths and are visualised using Filter Set C on a PE Biosystems ABI 377). Amplification reactions were performed in 10µl reaction mixes containing 1µM of forward primer and 1µM of reverse primer, 40ng DNA, 1x PCR buffer (PE Biosystems), 2.5mM Magnesium Chloride ($MgCl_2$), 0.8mM dNTP and 0.5 units of AmpliTaq Gold (PE Biosystems). The amplification conditions were 10 minutes at 95°C, followed by 8 cycles of 15 seconds at 95°C, 15 seconds at 50°C and 30 seconds at 72°C, followed by 27 cycles of 15 seconds at 89°C, 55 seconds at 55°C and 30 seconds at 72°C followed by 10 minutes at 72°C. 2.5µl of the 6-

FAM-labelled reaction, 2.5µl of the TET-labelled reaction and 10µl of the HEX-labelled reaction were pooled with 15µl of water so that the amplification products derived from the three primer pairs could be run in each lane of the gel. Samples were run out on polyacrilamide gels on an ABI 377 using standard conditions including an internal lane standard (GeneScan Reference Guide 1997). GeneScan 3.1 was used to analyse the results. Genographer (http://hordeum.oscs.montana.edu/genographer/) was used to display the results.

5.2.6 Bulk Segregant Analysis

As described previously, B-SA is when DNA from resistant individuals is pooled to form a resistant bulk and DNA from susceptible individuals is pooled to form a susceptible bulk (Michelmore *et al.*, 1991). Each bulk was made up of DNA from ten individuals to reduce background effects from unlinked fragments, ensuring that linked fragments were easily detectable.

AFLP Bulk Segregant Analysis

Following pre-selective amplification of DNA (GeneScan Reference Guide, 1997) extracted from each individual, the concentration of DNA for each sample was adjusted to 10ng/µl. Prior to selective amplification 50µl of pre-selectively amplified (preamped) DNA from each of ten resistant (or susceptible) individuals were combined to form a resistant (or susceptible) bulk. For line 22S AFLP B-SA, preamplified DNA from each of ten CDN 1 resistant backcross (B_1) individuals were pooled to form a resistant bulk, R1 and preamplified DNA from another ten CDN 1 resistant B_1 individuals were pooled to form a second resistant bulk, R2. Preamplified DNA from ten 22S CDN 1 susceptible B_1 individuals were pooled to form a susceptible bulk, S1, and preamplified DNA from another ten CDN 1 susceptible B_1 individuals were pooled to form a second susceptible bulk, S2. The bulks were selectively amplified with 52 primer pairs (Table 5.1) as described in section 5.2.4.

For line Jong Bai No. 2 preamplified DNA from ten UK 1 resistant B_1 individuals were pooled to form a resistant bulk, R6 and preamplified DNA from another ten UK 1 resistant individuals were pooled to form a second resistant bulk, R7. Preamplified DNA from ten UK 1 susceptible B_1 individuals were pooled to form a susceptible bulk, S6, and preamplified DNA from another ten UK 1 susceptible individuals were pooled to form a second susceptible bulk, S7. Fifty-three primer pairs were used to selectively amplify the bulks (Table 5.2) as described in section 5.2.4.

For each line, a primer pair was classed as polymorphic when a fragment was produced in the resistant bulks but was absent or present in lower amount in the susceptible bulks. Once a primer pair was identified as polymorphic through B-SA the original resistant parent, susceptible parent, F_1 and B_1 individuals were screened to determine if the fragment was linked to resistance. If a fragment is not linked to resistance 50% recombination will occur in the B_1 individuals. Recombination has occurred if a resistant B_1 individual lacks the fragment or if a susceptible individual has the fragment, such individuals are termed "recombinants." Thus, for a fragment to be linked to resistance, no more than 13 recombinants can be tolerated per 40 B_1 individuals tested because statistically this is significantly different from 50% recombination ($\chi^2=4.9$, $p<0.05$).

Once an AFLP primer pair was identified as polymorphic it was used to screen 30 individuals selected from the 72-8 *B. napus* mapping population (A. Sharpe, personal communication). Genomic DNA of these 30 individuals was obtained from A. Sharpe, AAFC, Saskatoon, Saskatchewan, Canada. The individuals were scored for the presence of the linked fragment (scored as "+") or absence of the linked fragment (scored as "-"). This scoring data enabled the AFLP marker to be

Table 5.1 Primer pair combinations used in AFLP analysis of line 22S resistant bulks (R1 & R2) and susceptible bulks (S1 & S2).

*Eco*RI primers	*Mse*I primers							
	CAA	**CAC**	**CAG**	**CAT**	**CTA**	**CTC**	**CTG**	**CTT**
AA	M	0	M	M	M	M	-	M
AC	M	P	P	0	M	M	M	M
AG	M	0	0	-	0	-	M	0
AT	-	0	M	0	M	M	-	M
TA	0	0	M	M	0	0	M	M
TC	P	*P*	-	-	M	-	M	M
TG	0	M	M	0	-	-	0	-
TT	0	0	M	0	0	M	M	-

Notes:
- M Primer pair produced monomorphic amplification pattern
- \- Primer pair was not used
- 0 Primer pair failed to produce amplification product
- P Primer pair produced AFLP band that was present in resistant bulks but was absent from susceptible bulks. Italicised represents primer pair combination that produced an AFLP product that was linked to resistance

Table 5.2 Primer pair combinations used in AFLP analysis of line Jong Bai No. 2 UK 1 resistant bulks (R6 & R7) and UK 1susceptible bulks (S6 & S7).

*Eco*RI primers	*Mse*I primers							
	CAA	**CAC**	**CAG**	**CAT**	**CTA**	**CTC**	**CTG**	**CTT**
AA	P	M	M	M	0	M	-	M
AC	M	P	P	M	M	P	M	M
AG	0	0	-	-	0	-	P	0
AT	-	M	M	0	M	M	0	M
TA	0	0	M	0	0	0	M	M
TC	M	-	P	-	P	-	M	M
TG	0	0	0	P	0	-	M	-
TT	0	0	0	0	0	M	M	-

Notes:
- M Primer pair produced monomorphic amplification pattern
- \- Primer pair was not used
- 0 Primer pair failed to produce amplification product
- P Primer pair produced AFLP band that was present in resistant bulks but was absent from susceptible bulks.

associated to a linkage group on the genetic linkage map. Once the AFLP marker was positioned on a linkage group, microsatellites from that linkage group (see below) were used to produce a mapping interval in which the resistance allele was located.

Microsatellite Bulk Segregant Analysis

For line 22S, genomic DNA from the same individuals used in AFLP B-SA were pooled to produce bulks R1, R2, S1 and S2 for microsatellite bulk segregant analysis. Thirty-three microsatellite primers from B. napus linkage group N6 were used to screen the bulks, genomic DNA from the resistant 22S individual, N-o-1 and the 22S F1, as described in section 5.2.5.

For line 165, genomic DNA from ten UK 1 resistant B1 individuals were pooled to form a resistant bulk, R3, and DNA from ten UK 1 susceptible B1 individuals were pooled to form a susceptible bulk, S3. Genomic DNA from ten CZE 1 resistant B1 individuals were pooled to form a resistant bulk, R4 and genomic DNA from another ten CZE 1 resistant B1 individuals were pooled to form another resistant bulk, R5. Genomic DNA from ten CZE 1 susceptible B1 individuals were pooled to form a susceptible bulk, S4 and genomic DNA from another ten CZE 1 susceptible B1 individuals were pooled to form a second susceptible bulk, S5. All 165 bulks and genomic DNA from individuals 165, N-o-9 and 165 F1 were screened with 447 microsatellite primers, from all linkage groups on the B. napus genetic linkage map, using conditions described in section 5.2.5.

For line Jong Bai No. 2 microsatellite B-SA, genomic DNA from the same individuals used in AFLP B-SA were pooled to produce bulks R6, R7, S6 and S7. Microsatellite primers from B. napus linkage group N6 were used to screen the bulks and genomic DNA from the resistant Jong Bai No. 2 individuals, R-o-18 and Jong Bai No. 2 F1 using the conditions described in section 5.2.5.

As for AFLP B-SA, a microsatellite primer pair was classed as polymorphic when a fragment was amplified in both resistant bulks but was amplified less or failed to amplify in the susceptible bulks. Once a primer pair was identified as polymorphic through B-SA, the original resistant parent, susceptible parent, F1 and B1 individuals were screened to determine if the fragment was linked to resistance. Genomic DNA was extracted from additional 165 B1 individuals, using the Kirby Mix technique described in section 5.2.2 and were included in the screen of 165 B1 individuals using polymorphic microsatellites. As for AFLP, if 50% recombination occurred, a fragment that appeared to be linked to resistance by B-SA was deemed to be unlinked to resistance.

5.2.7 MapMaker

Once linked AFLP and microsatellite markers, that mapped to the same linkage group, were established a computer software package, MapMaker (Lander *et al.*, 1987), was used to generate a linkage map with the interval in which the TuMV resistance allele mapped.

5.3 RESULTS

5.3.1 Line 22S

AFLP Bulk Segregant Analysis

Of the 52 primer pairs used to screen line 22S resistant and susceptible bulks, 19 failed to produce product and 27 produced monomorphic amplification band patterns (Table 5.1). AFLP bulk segregant analysis successfully identified one primer pair (*Eco*RI-TC, *Mse*I – CAC, Table 5.1) that produced a clear polymorphism, with a band present between 91bp and 92bp in bulks R1 and R2 which was less intense in bulks S1 and S2 (Figure 5.1). Thirty-seven out of the 40 B_1 individuals screened with the primer pair produced amplification product. Six of the 37 individuals that produced product were identified as recombinants on the basis that a fragment of the correct size was not produced in two resistant individuals but was amplified in four susceptible individuals. This primer pair was also polymorphic between mapping parents N-o-1 and N-o-9, where a fragment between 91bp and 92bp was produced in N-o-9 that was not amplified in N-o-1 (Figure 5.2). A further three primer pairs were polymorphic between the 22S bulks (Table 5.1) but they did not amplify product of the relevant size in mapping parent N-o-9.

Anchoring the resistance gene to a linkage group

The AFLP marker, now called EtcMcac1, linked to TuMV CDN 1 resistance in line 22S was positioned on the *B. napus* gentic map on linkage group N6 (Figure 5.3) by screening 30 individuals from the 72-8 mapping population with primer pair combination *Eco*RI - TC, *Mse*I - CAC.

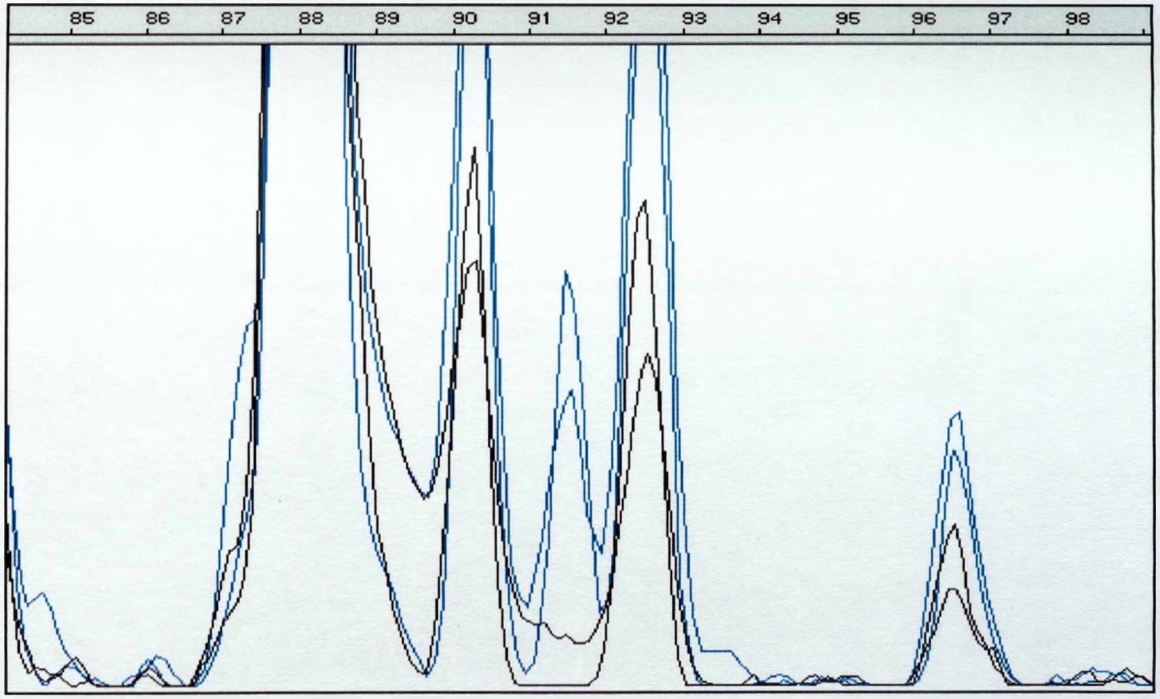


Figure 5.1 AFLP bulk segregant analysis of line 22S CDN 1 resistant and susceptible bulks with AFLP primer pair combination *Eco*RI-TC and *Mse*I-CAC. Blue traces represent resistant bulk AFLP pattern; black traces represent CDN 1 susceptible bulks AFLP pattern. Fragment of interest is between 91bp and 92bp.

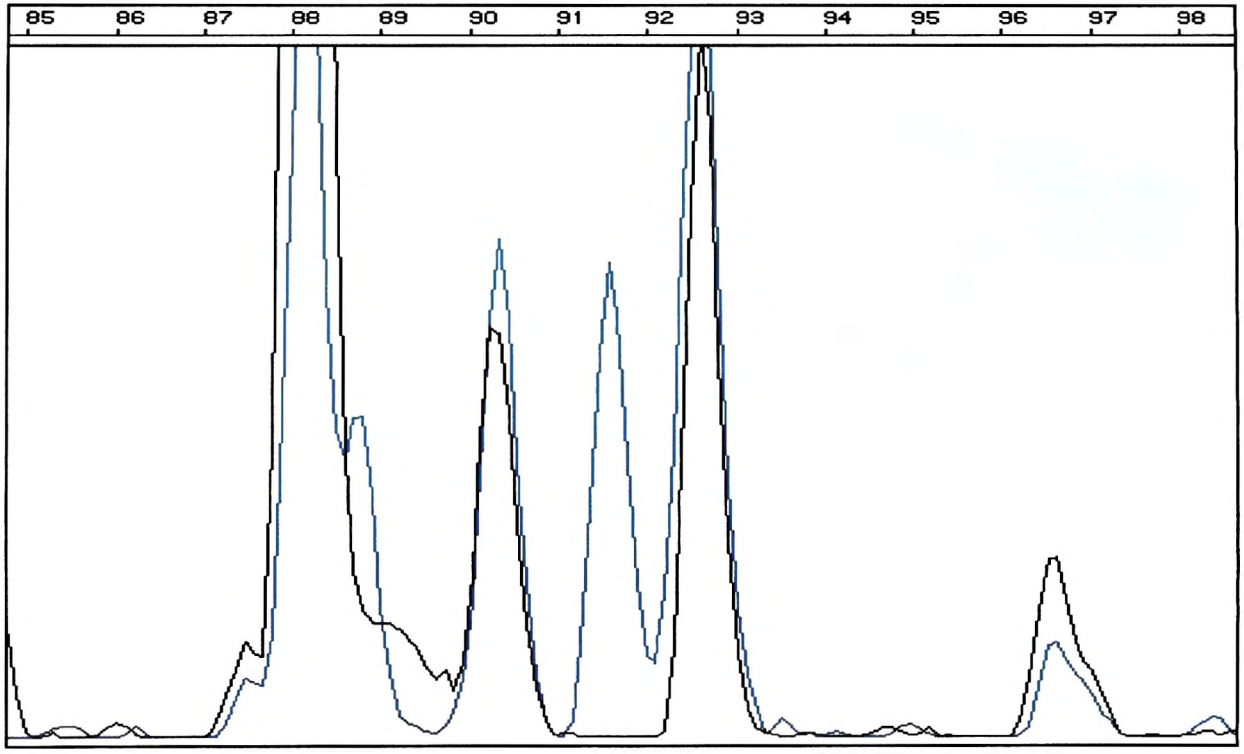


Figure 5.2 AFLP bulk segregant analysis of lines N-o-1 and N-o-9 with AFLP primer pair combination *EcoRI*-TC and *Mse1*-CAC. Blue trace represent N-o-9 AFLP pattern; black trace represent N-o-1 AFLP pattern. Fragment of interest is between 91bp and 92bp.

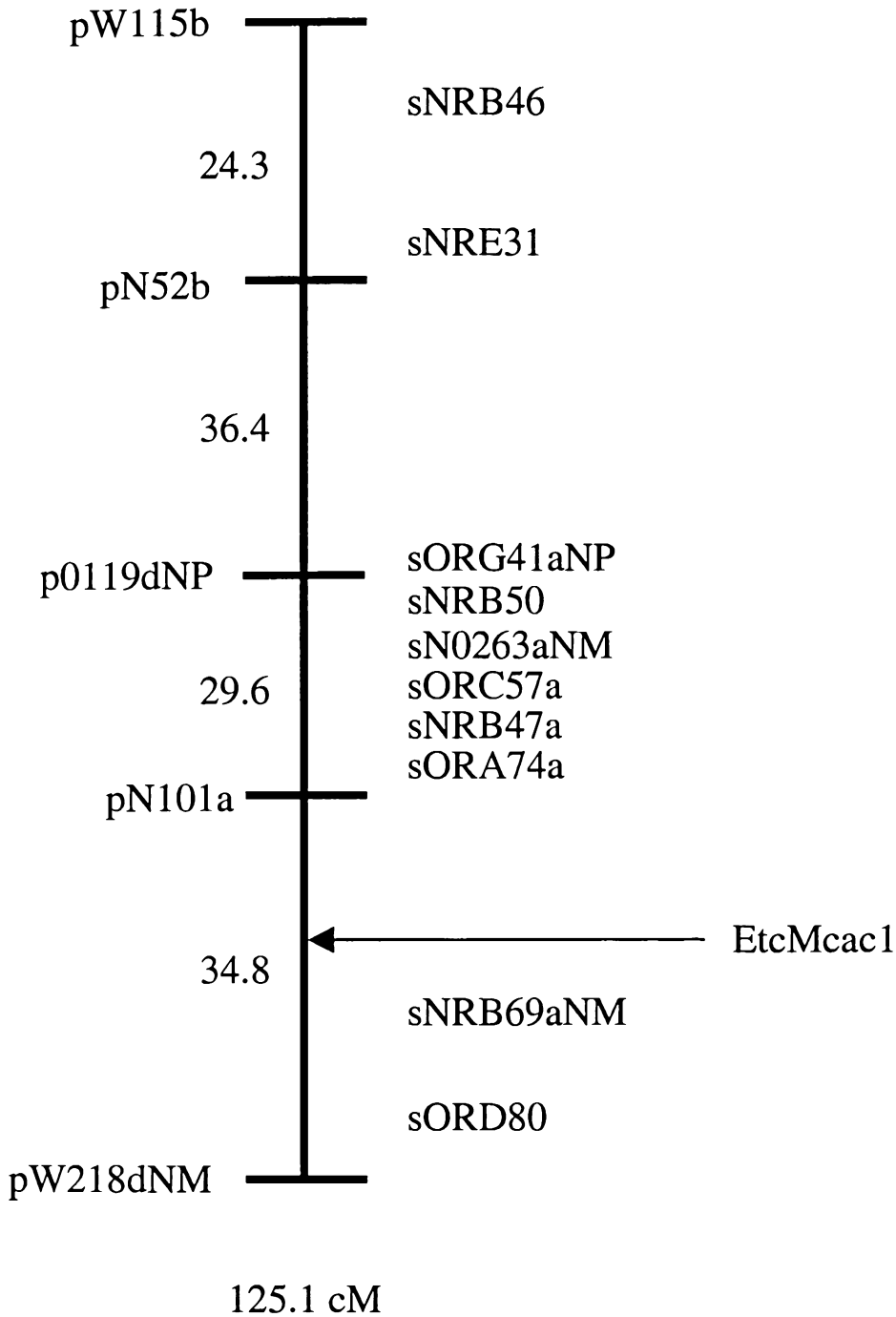


Figure 5.3 Position of AFLP marker, EtcMcac1, linked to CDN 1 resistance in line 22S, on *B. napus* linkage group N6. RFLP defined loci are on the left; microsatellite defined loci are on the right. Distances between RFLP defined loci are in centiMorgans (cM) on the left hand side of the diagram and the total length of the linkage group, 125.1cM, is shown at the bottom.

Six of the 33 Microsatellite primers from linkage group N6 produced polymorphic amplification products. Of the microsatellites used, a further seven did not give product and 20 gave products that were monomorphic. Examples of a monomorphic microsatellite, a microsatellite that was polymorphic between the parents but not the bulks and one that was polymorphic between the parents and also polymorphic between the bulks are shown in Figure 5.4.

The individuals that made up the bulks were screened with the six polymorphic microsatellite primers, see Figure 5.5 for an example of a mapping gel. The scoring data (Figure 5.6) allowed the dominant 22S TuMV resistance allele to be positioned on linkage group N6 between microsatellite markers sNRB93 (7.6cM) and sS1949 (15.5cM), see Figure 5.7. The gene conferring resistance to CDN 1 has been named *TuRB03* (*TuMV Resistance in Brassica 03*).

5.3.2 Line 165

Microsatellite Bulk Segregant Analysis

Four hundred and forty-seven microsatellite primers were used to screen 165, N-o-9, F_1, UK 1 resistant bulk, R3, UK 1 susceptible bulk, S3, CZE 1 resistant bulks, R4 and R5 and CZE 1 susceptible bulks, S4 and S5. Two hundred and twenty nine of the microsatellite primers produced monomorphic band patterns and 55 did not give any product. Seventy-eight microsatellite primers produced a band from N-o-9 but not from 165. Seventy-one primers produced a band from 165 but not from N-o-9. None of these were classed as polymorphic because the band was present in all the bulks. A further 14 primers produced fragments that were amplified from 165 but not from N-o-9 that were also amplified in the resistant bulks but not the susceptible bulks; 12 of these were found not to be linked to resistance when tested on individuals.

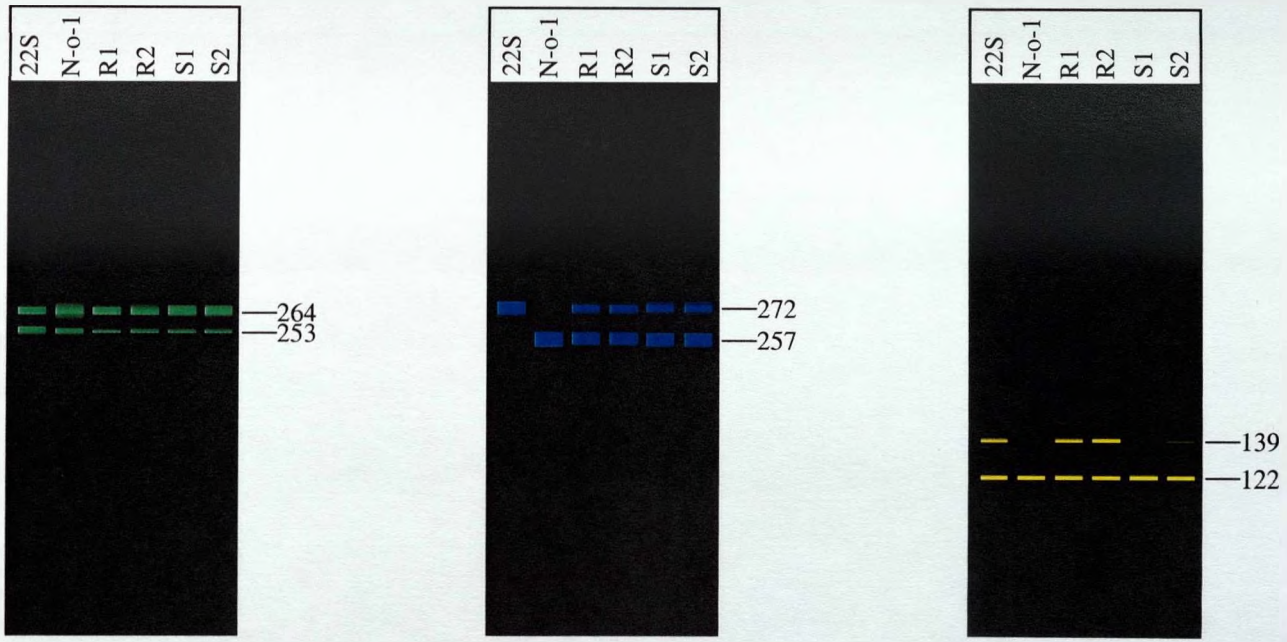


Figure 5.4 Microsatellite analysis of lines 22S and N-o-1, and Bulk Segregant Analysis of CDN 1 resistant bulks (R1 & R2) and CDN 1 susceptible bulk (S1 & S2). Examples of (a) monomorphic pattern produced by microsatellite sN0263 (b) a pattern at 272bp that was polymorphic between lines 22S and N-o-1, but was monomorphic between R1, R2, S1 and S2 produced by microsatellite sNRE31 (c) a polymorphic pattern at 139 bp between lines 22S, N-o-1 and bulks produced by microsatellite sNRB93.

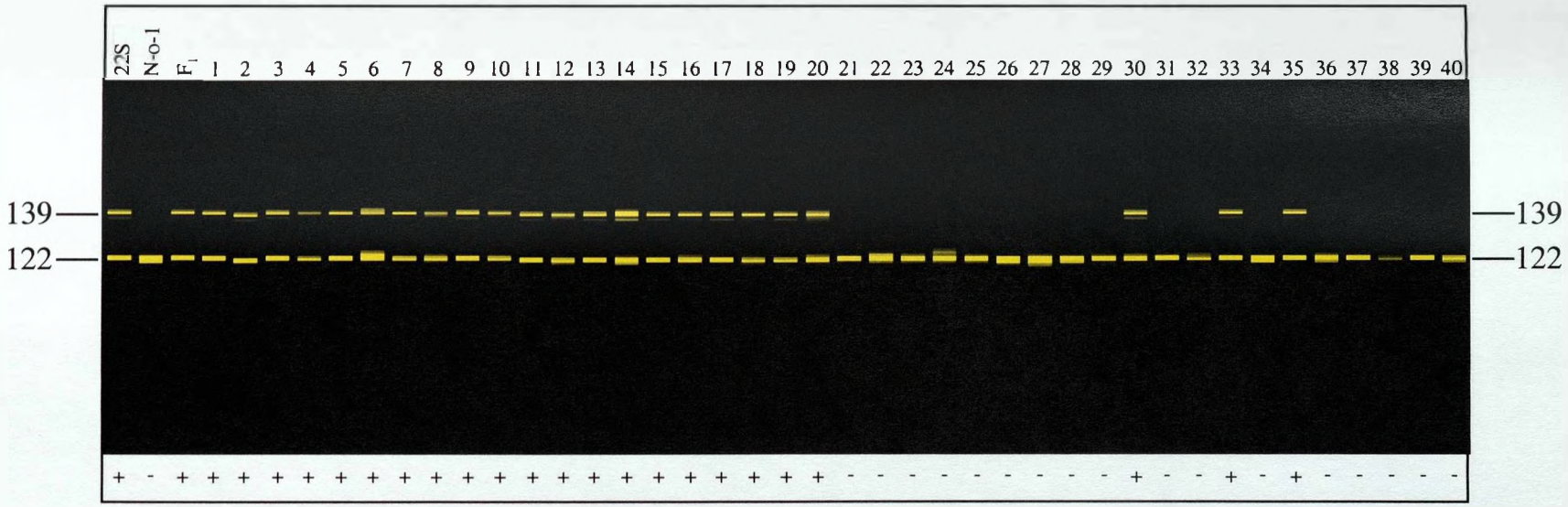


Figure 5.5 Microsatellite analysis of line 22S B_1 individuals with primer sNRB93. 1-20, CDN 1 resistant B_1 individuals from bulks R1 and R2; 21-40, CDN 1 susceptible B_1 individuals from bulks S1 and S2. +, presence of band of interest, 139bp; -, absence of band of interest.

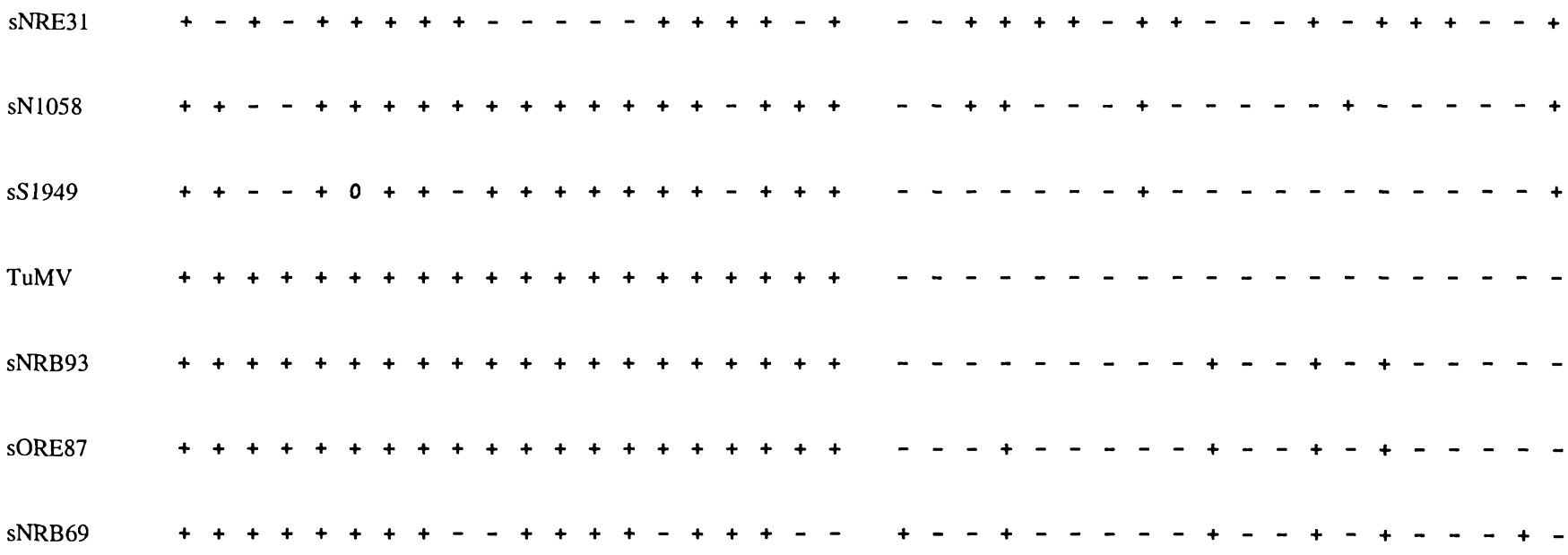


Figure 5.6 Scoring data for microsatellites, from linkage group N6, that were polymorphic through bulk segregant analysis on 22S B_1 resistant and susceptible bulks. Rows indicate microsatellite primers, except row labelled "TuMV" which indicates a hypothetical microsatellite coincident with the resistance allele. Columns correspond to B_1 individuals (1-20 are resistant to TuMV isolate CDN 1 and 21-40 are susceptible to CDN 1). +, presence of 22S allele; -, presence of N-o-1 allele; 0, no product amplified.

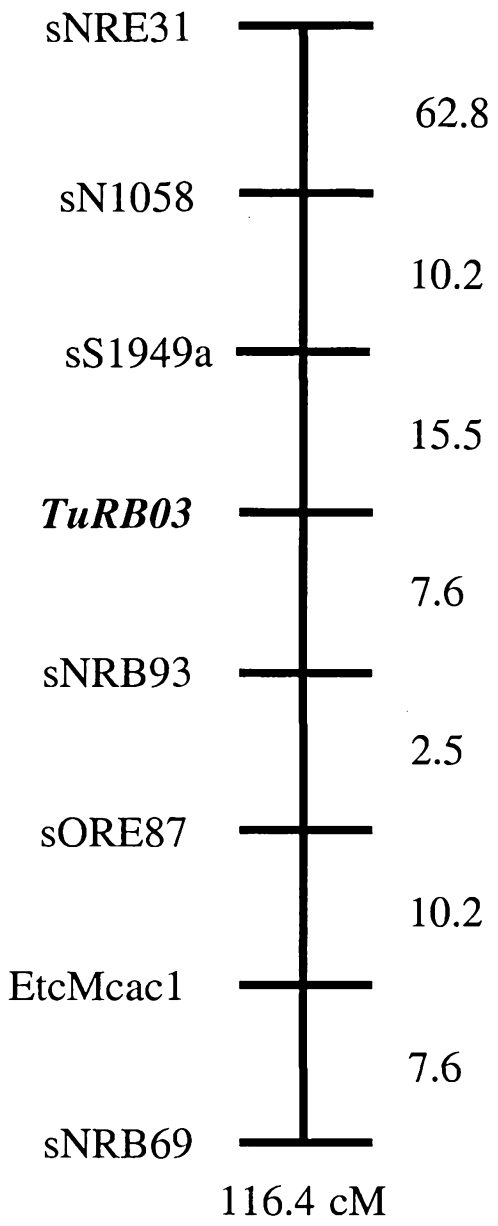


Figure 5.7 Linkage group N6 with microsatellite defined loci and one AFLP-defined loci (EtcMcac1). Map distances are in centiMorgans (cM) on the right hand side of the linkage group with the total length of the linkage group at the bottom, 116.4cM. All loci separated by recombination are represented with even spacing. The resistance locus *TuRB03* was 7.6cM from sNRB93 and 15.5cM from sS1949.

Microsatellite sNRE31 was interesting because it maps solely to linkage group N6, the linkage group where the previously mapped TuMV resistance genes *TuRB01* and *TuRB01b* are located (Walsh *et al.*, 1999; Rusholme, 2000). Microsatellite sNRE31 appeared to be linked to UK 1 resistance through B-SA (Figure 5.8), and when observed at the individual level five out of 20 individuals were recombinants (Figure 5.9), which is significantly different from 50% recombination (χ^2=5.00, p<0.05). Primer sNRE31 did not appear to be linked to CZE 1 resistance at the bulk level (Figure 5.8) or at the individual. Seventy-two B_1 individuals were screened with microsatellite primer sNRE31 (see Figure 5.9 for gel image of a subset of these individuals). Thirty-one out of 72 B_1 individuals were identified as recombinants, which is not significantly different from 50% recombination (χ^2=1.658, p>0.05).

Results from B-SA with microsatellite primer sN0202 suggest that it was linked to CZE 1 resistance but not UK 1 resistance (Figure 5.8). The scoring data at the individual level supported this. Seven out of 20 B_1 individuals, inoculated with UK 1, were identified as recombinants (Figure 5.10), which is not significantly different from 50% recombination (χ^2=1.800, p>0.05). The scoring data for B_1 individuals, inoculated with CZE 1, suggest that microsatellite sN0202 could be linked to resistance. Eighteen of 72 B_1 individuals were identified as recombinants (see Figure 5.10 for gel image of a subset of the individuals), which is significantly different from 50% recombination (χ^2=18.00, p<0.05).

5.3.3 Line Jong Bai No 2

AFLP Bulk Segregant Analysis

Of the 53 primer pairs used for Jong Bai No. 2 AFLP B-SA, 21 did not produce amplified product and 24 produced monomorphic band patterns (Table 5.2). One primer pair (*Eco*RI-TC, *Mse*I-CTA) was polymorphic between the bulks (Figure 5.11). When the UK 1 resistant and susceptible B_1 individuals were amplified

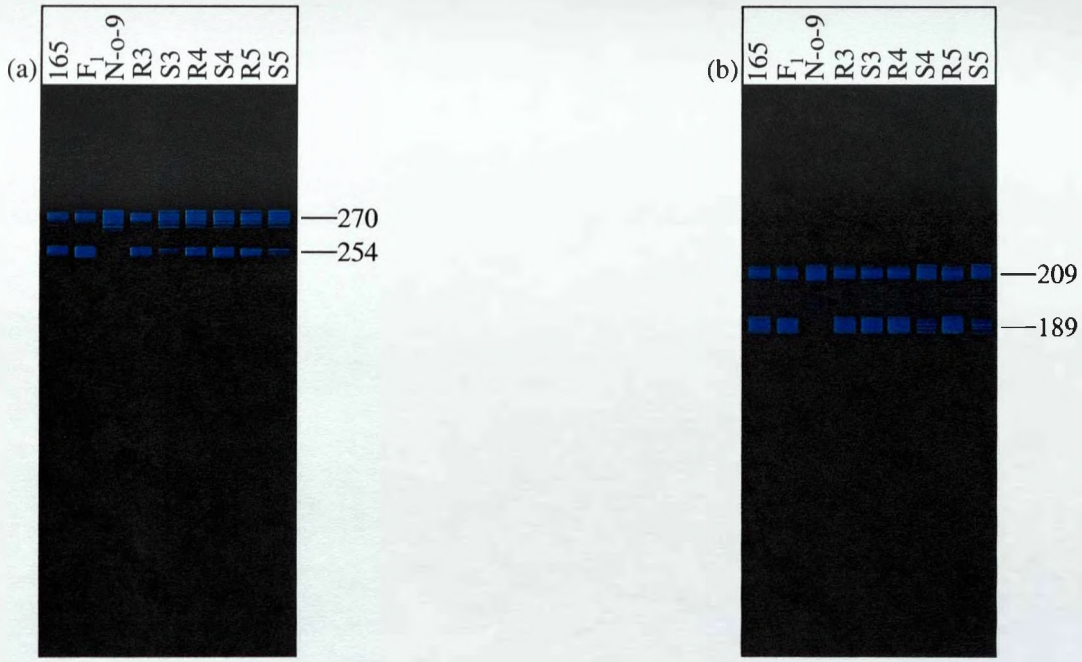
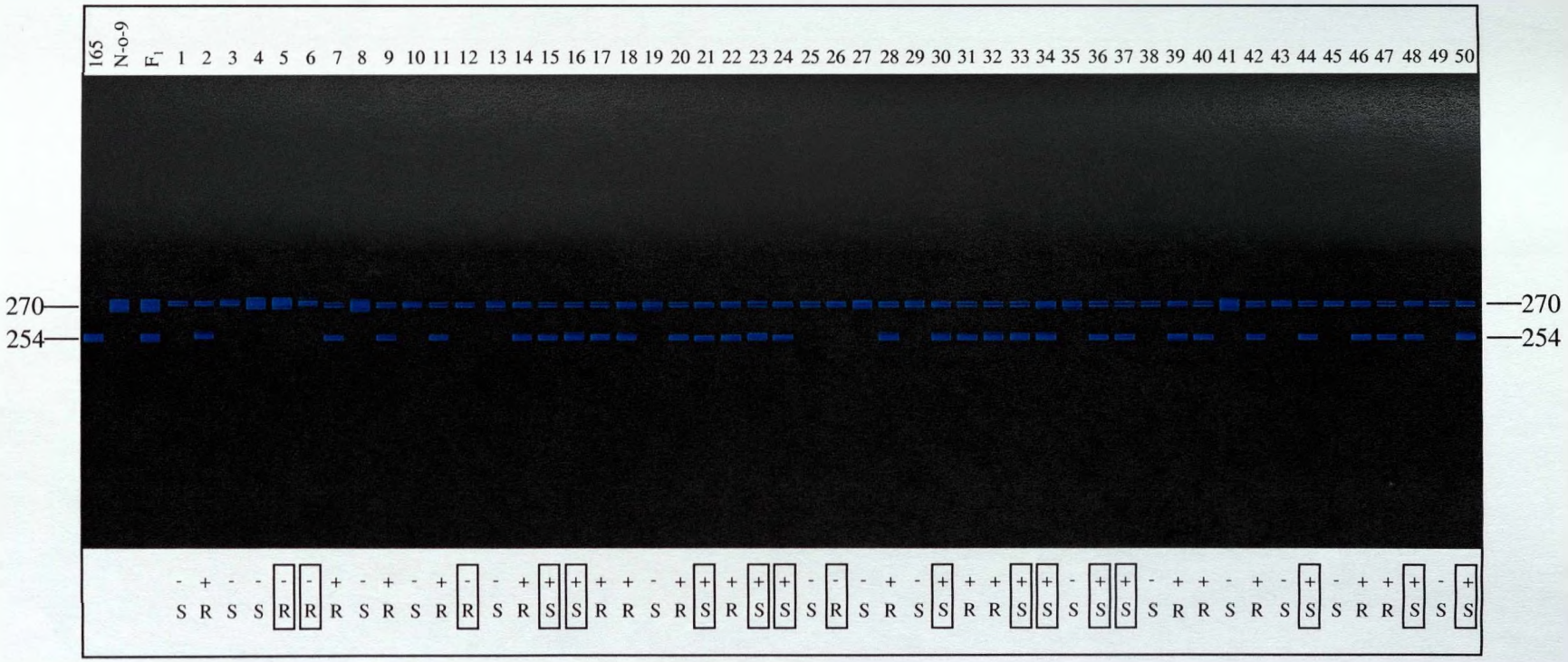


Figure 5.8 Microsatellite analysis of 165, F_1, N-o-9, Bulk Segregant Analysis of UK 1 resistant bulk (R3), CZE 1 resistant bulks (R4 & R5), UK 1 susceptible bulk (S3) and CZE 1 susceptible bulks (S4 & S5) with microsatellites (a) sNRE31 and (b) sN0202 (rhs)


165
N-o-9
F1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
270
254
270
254
- + - - - - + - + - + - - + + + + - + + + + + - + + + + - + + + + -

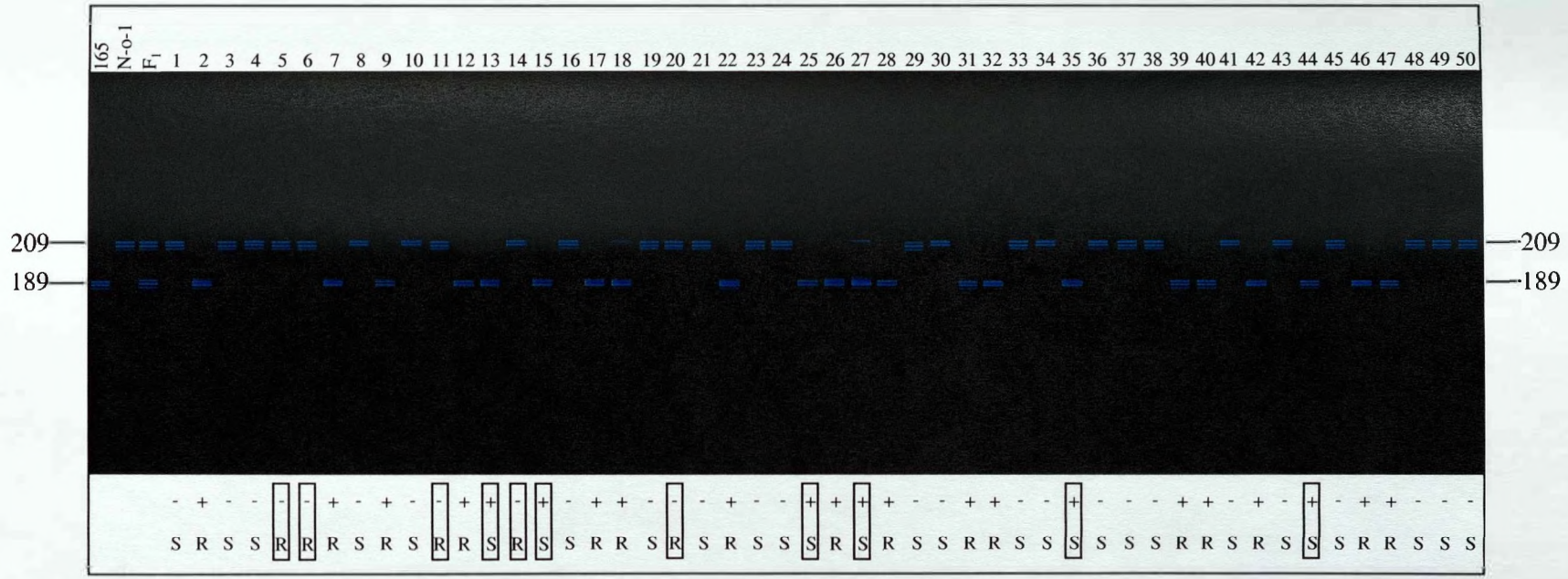


Figure 5.10 Microsatellite analysis of line 165 B_1 individuals with primer sN0202. 1-20, B_1 individuals inoculated with TuMV isolate UK 1; 21-50, B_1 individuals inoculated with TuMV isolate CZE 1. R, TuMV resistant individuals; S, TuMV susceptible individuals. +, presence of the fragment of interest (189bp, maps to N8); -, absence of fragment of interest. Recombinants are shown in boxes

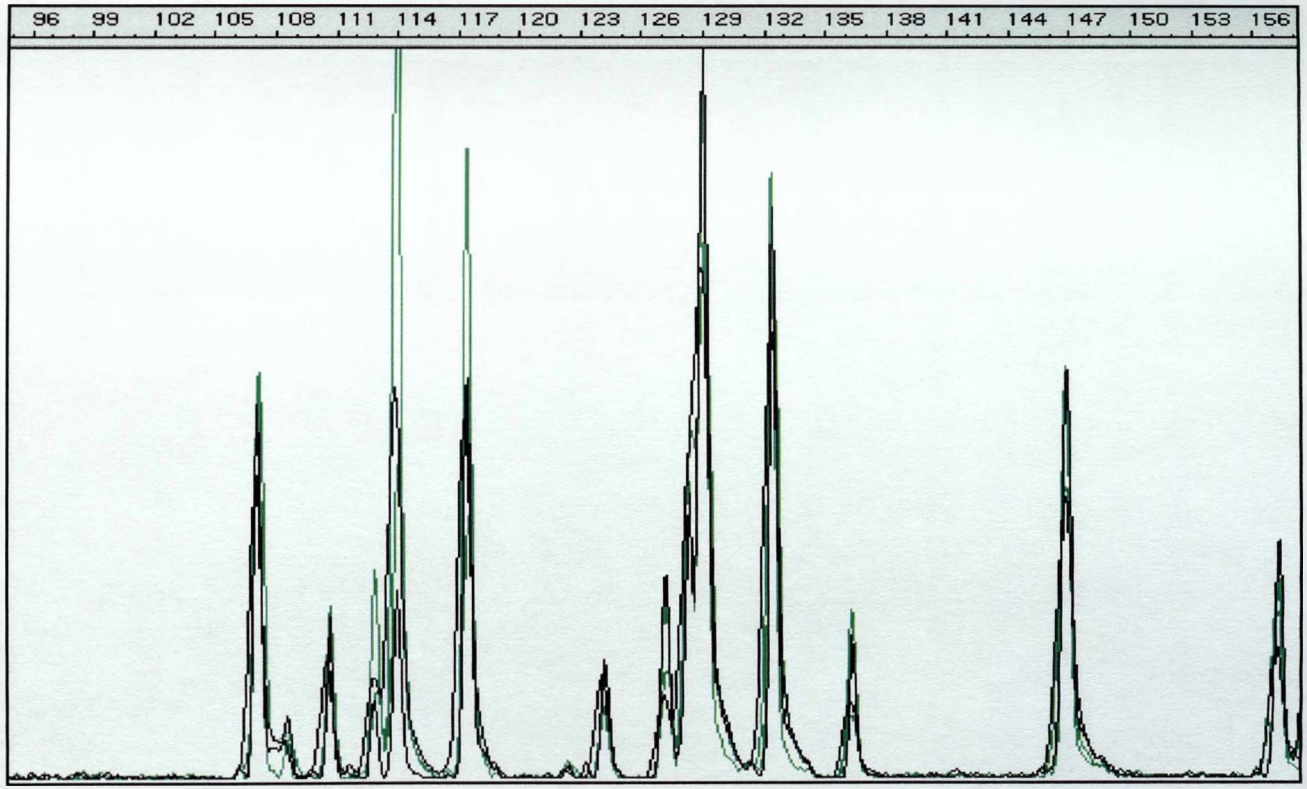


Figure 5.11 AFLP bulk segregant analysis of line Jong Bai No. 2 UK 1 resistant and susceptible bulks with primer pair combination. Green traces represent UK 1 resistant bulks AFLP patterns;black traces represent UK 1 susceptible bulks AFLP patterns. Fragment of interest is at 114bp.

with the primer pair, 12 of the 40 individuals were identified as recombinants, which is significantly different from 50% recombination (χ^2=6.40, p>0.05). Another seven primer pairs produced polymorphic band patterns in the bulks (Table 5.2) but were not used on the individuals or mapping parents. I attempted to analyse these polymorphic primer pairs on Jong Bai No. 2 B_1 individuals at AAFC, Saskatoon, Canada, but PCR amplification was unsuccessful. Amplification was also unsuccessful when attempting to anchor the marker linked to resistance in Jong Bai No. 2, produced by primer pair *Eco*RI-TC and *Mse*I-CTA, on a *B. rapa* mapping population (A. Sharpe, personal communication).

Microsatellite Bulk Segregant Analysis

Failure of amplification of AFLP product meant an alternative strategy had to be used to attempt the mapping of the dominant allele conferring resistance to UK 1 in Jong Bai No. 2. Microsatellites were used through B-SA to identify markers linked to resistance in Jong Bai No. 2. Due to lack of time, only microsatellites from linkage group N6 were used to screen Jong Bai No. 2 resistant individual, R-o-18, F_1 and bulks to determine if the resistance allele mapped in the same region as *TuRB01* and *TuRB01b* (Walsh *et al.*, 1999; Rusholme, 2000).

Figures 5.12 and 5.13 show that five primer pairs from the upper region of linkage group N6 were polymorphic with bands being produced in Jong Bai No. 2, F_1 and resistant bulks that were absent from R-o-18 and absent or less intense in susceptible bulks. Microsatellite sNRE31 is particularly interesting because this microsatellite only maps to the top of linkage group N6.

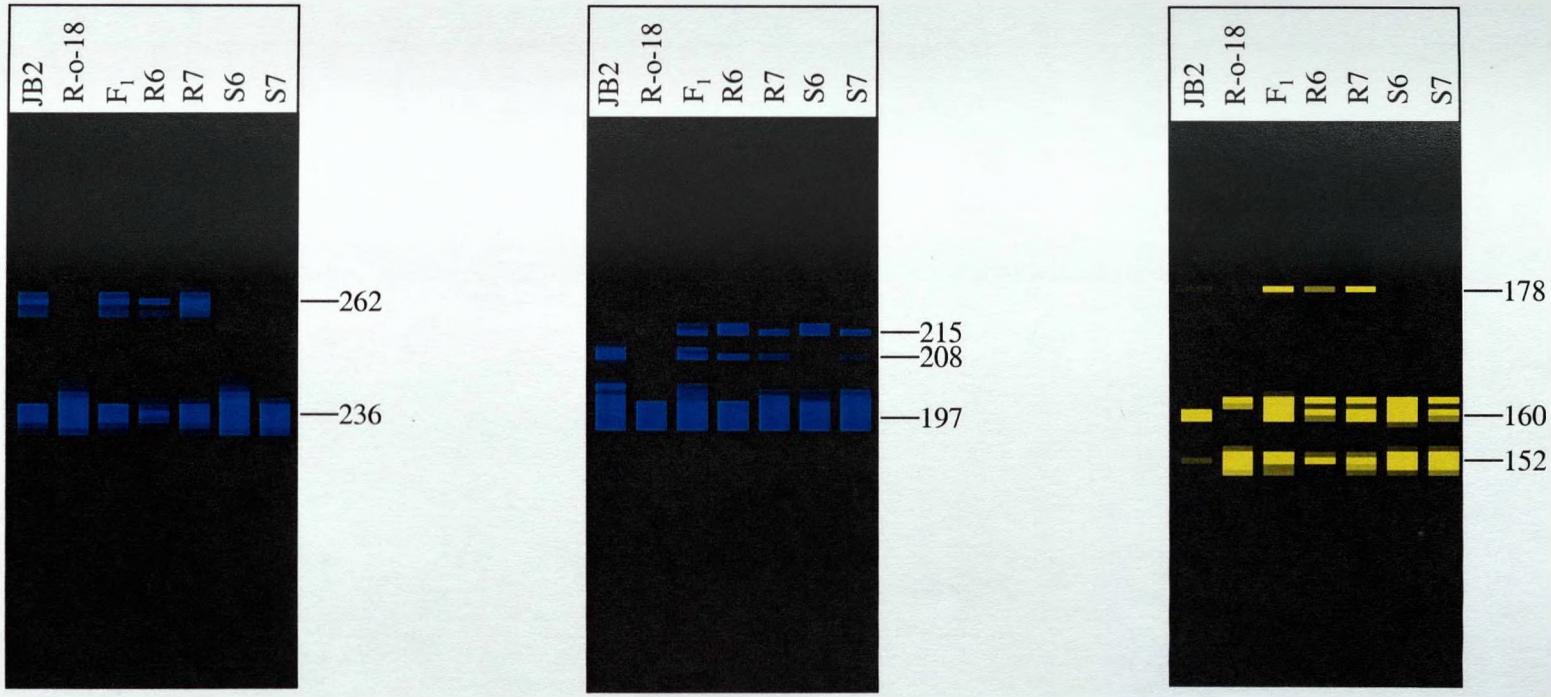


Figure 5.12 Microsatellite analysis of Jong Bai No. 2 (JB2), R-o-18, F1 and Bulk Segregant Analysis of UK 1 resistant bulks (R1 & R2) and UK 1 susceptible bulks (S1 & S2) with primers: (a)sNRE31, band of interest at 262bp, maps to N6 (b)sORC57, band of interest at 208bp, maps to N6 and (c)sS2165, band of interest at 178bp, maps to N6.

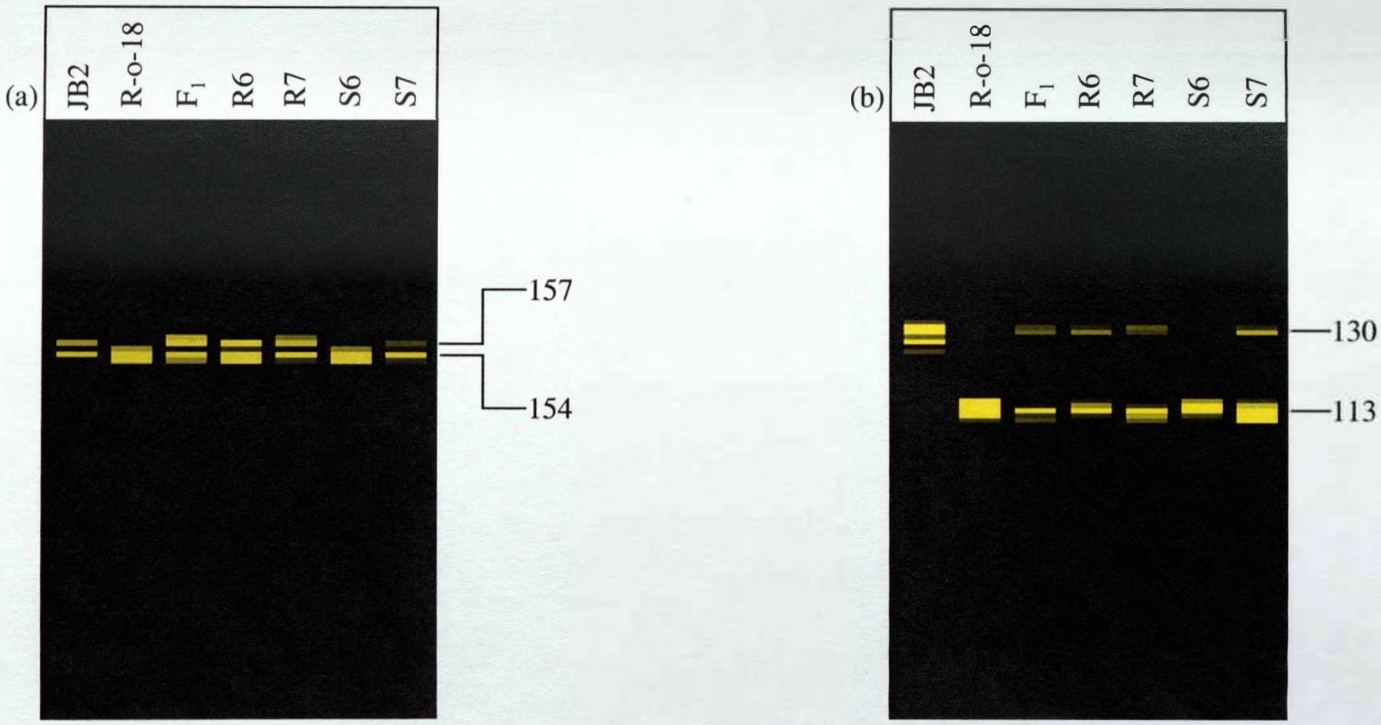


Figure 5.13 Microsatellite analysis of Jong Bai No. 2 (JB2), R-o-18, F1 and Bulk Segregant Analysis of UK 1 resistant bulks (R1 & R2) and UK 1 susceptible bulks (S1 & S2) with primers: (a)sN2837, band of interest at 157bp, maps to N6 and (b)sORE87, band of interest at 130bp, maps to N6.

5.4 DISCUSSION

The molecular analysis of TuMV resistance in *B. napus* line 22S has confirmed the conclusion from genetic analysis that a single dominant allele confers resistance to pathotype 4 isolate CDN 1 (Chapter 4). The dominant allele, now named *TuRB03*, has been successfully positioned in the lower region of linkage group N6 using AFLP and microsatellite bulk segregant analysis. The position of *TuRB03* coincides with two previously mapped TuMV resistance genes, *TuRB01* and *TuRB01b* (Walsh *et al.*, 1999; Rusholme, 2000).

The success of the B-SA approach is dependent upon the genetic divergence between the parents in the target region and the approach is more suited to outbreeding species (Michelmore *at al.*, 1991). AFLP and microsatellite B-SA revealed low levels of polymorphisms between line 22S and line N-o-1, suggesting these lines are closely related in the region of interest. Both lines are *B. napus* (oilseed rape) which is an inbreeding species. Also, line N-o-1 has resistance to TuMV isolate UK 1 (see Table 4.2, Chapter 4) conferred by the mapped resistance gene *TuRB01* (Walsh *et al.*, 1999). The similarity in the phenotypes conferred by *TuRB01* and *TuRB03* to TuMV isolates UK 1 and CDN 1 respectively and their similarity in map position, suggest they could have evolved from the same ancestor, so the regions in which the resistance genes are located may not be diverged. Similarly, results from 165 microsatellite B-SA identified low numbers of polymorphisms, with two microsatellites out of 447 linked to TuMV resistance, between line 165 (*B. napus*, swede) and line N-o-9 (*B. napus*, oilseed rape). Hence, although B-SA was successful in identifying markers linked to resistance it may not have been the best technique to use.

Alternatively, the low number of polymorphisms observed between the 165 bulks suggests that the control of TuMV resistance is more complex than the single dominant allele model proposed in Chapter 4. Data from analysing the avirulence determinant in TuMV isolate UK 1 for line 165 indicates that at least two mutations are required for UK 1 to fully break the resistance on line 165 (C.

Jenner, personal communication). Jenner *et al.* (2000) identified that the CI protein of TuMV was the avirulence determinant for the dominant resistance gene *TuRB01*, which is consistent with a typical gene-for-gene type interaction. Hence, if a gene-for-gene interaction occurred between UK 1 and 165 at least two resistance alleles are required for resistance to UK 1, which would explain the low number of polymorphisms between bulks. No analysis of the avirulence determinant of CZE 1 has been attempted.

The preliminary results from mapping "dominant allele(s)" conferring resistance to TuMV isolates UK 1 and CZE 1 in line 165 suggest that the resistances may be controlled by separate genes and that these genes map in separate locations. Microsatellite sNRE31 only maps to the upper region of linkage group N6 and this microsatellite appeared to be weakly linked to the UK 1 resistance. Microsatellite sN0202 appeared to be linked to the dominant allele conferring apparent immunity to CZE 1, but it was unclear if the microsatellite mapped to N8 (A-genome) or N18 (C-genome). Further molecular analysis would be required to confirm whether the linked microsatellite sN0202 mapped to N8 or N18. Preliminary RFLP analysis suggests that the allele may be located on linkage group N8 (R. Rusholme, personal communication). If the dominant allele conferring apparent immunity to CZE 1 maps to N8 it could coincide with another dominant resistance gene, *ConTR01*, that is part of a multigenic system providing broad spectrum TuMV resistance in *B. rapa* (Rusholme, 2000). If it maps to N18 this would be the first example of a major TuMV resistance gene mapping to the C-genome. Only a QTL for TuMV resistance, *TuRB02*, has previously been mapped to the C-genome (Walsh *et al.*, 1999). If the resistance maps to the C-genome long-term objectives such as transferance of resistance genes into TuMV susceptible, agronomically important *B. oleracea* crops would be easier.

Molecular analysis of the resistance to UK 1 in *B. rapa* line Jong Bai No. 2 provided evidence for the single dominant allele genetic model proposed in Chapter 4. Microsatellite B-SA suggested that the dominant allele (*TuRB05*) was positioned at the upper region of linkage group N6. The mapped resistance genes

TuRB01 and *TurB01b*, which are also resistant to UK 1 are located at the bottom of linkage group N6 (Walsh *et al.*, 1999; Rusholme, 2000). Jong Bai No. 2 had a different specificity of interaction to *TuRB01* and *TuRB01b* because it had resistance to UK 1M in addition to resistance to UK 1 (Chpater 4). As described in Chapter 4, UK 1M overcomes both *TuRB01* (Lehmann *et al.*, 1997) and *TuRB01b* (Rusholme, 2000). It is possible that the allele conferring apparent immunity to UK 1 is related to *TuRB01* and *TuRB01b.* An evolutionary event, e.g. point mutation(s), may have occurred to change specificity and another evolutionary event e.g. a translocation event, may have altered the genomic position of the resistance allele, as described by Holub (2001). The occurrence of an RFLP marker common to both regions (R. Rusholme, personal communication) may indicate the translocation event to be true. Further work on B_1 individuals, future generations and mechanism of action of TuMV resistance genes would have to be pursued to prove/disprove these suggestions.

Figure 5.14 summarises the positions of TuMV resistance alleles mapped and roughly mapped in this project, along with previously mapped alleles (Walsh *et al.*, 1999; Rusholme, 2000). Three resistance alleles, *TuRB01* (Walsh *et al.*, 1999), *TuRB01b* (Rusholme, 2000) and *TuRB03* (see above), map in the same region of linkage group N6. The Jong Bai No. 2 and 165 UK 1 resistances may map to a different region of N6. Also, an allele (*TuRB04*) conferring resistance to CZE 1 in 165 could be located on N8 where another TuMV resistance allele, *CoNTR01*, is located (Rusholme, 2000). These results suggest that evolution of resistance gene clusters may apply for TuMV resistance. It would be interesting to find out about the homologies between linkage groups N6 and N8 to see if there are common markers that may suggest common evolutionary patterns between the resistance genes located on different linkage groups.

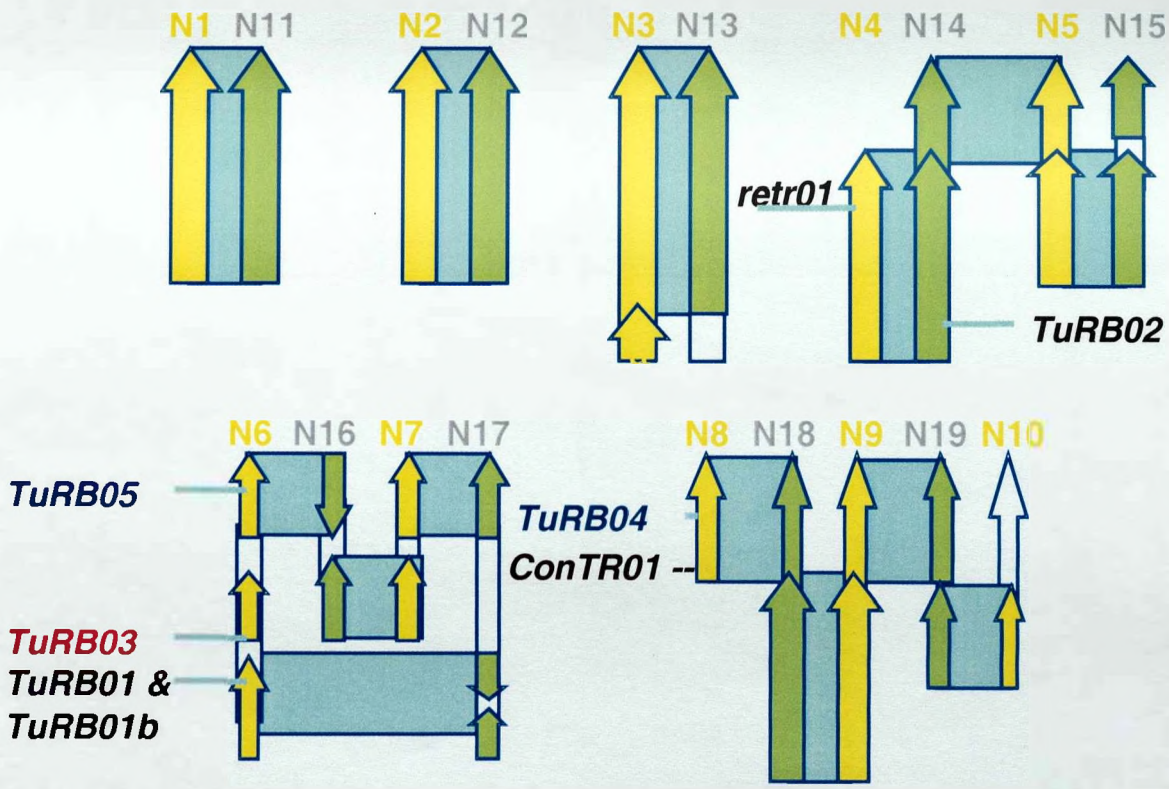


Figure 5.14 Summary of mapped and roughly mapped TuMV resistance genes. N1-N10, A-genome (*B. rapa*) linkage groups; N11-N19, C genome (*B. oleracea*) linkage groups. Genes shown in black are previously mapped resistance genes (Walsh *et al.*, 1999; Rusholme, 2000). Gene shown in red (*TuRB03*) was mapped during this project. Genes shown in blue (*TuRB04* and *TuRB05*) were roughly mapped during this project.

CHAPTER 6

GENERAL DISCUSSION

The first aim of this project was to develop experimental techniques for screening *A. thaliana* ecotypes for resistance to TuMV. This was completed successfully and several *A. thaliana* ecotypes were screened with five TuMV isolates. Unfortuantely, no TuMV resistance was identified in this study.

The second aim of this study was to identify *Brassica* lines that were resistant to isolates representing the three most common pathotype of TuMV (1, 3 and 4), lines that could differentiate between two pathotype 3 isolates and any lines that had novel specificities of interaction. This was completed successfully with 33 sources of resistance being identified, some of which were resistant or apparently immune to all isolates used, some could differentiate between the pathotype 3 isolates and some had new specificities of interaction. The resistance phenotype suggests that resistance acts to prevent virus entry into phloem sieve elements (Figure 6.1a). Alternatively, the virus may be able to enter the sieve elements but is unable to exit them into phloem parenchyma/companion cells (Figure 6.1b). The apparent immunity phenotype suggests that virus replication or cell-to-cell movement is restricted (Figure 6.2). The TuMV resistance gene *TuRB01* confers apparent immunity against UK 1 (Walsh *et al.*, 1999). Mutations of the TuMV-CI protein enabled UK 1 to become virulent on *TuRB01* plants (Jenner *et al.*, 2000). The mutations were not reported to interfere with the helicase activity or cell-to-cell movement of the CI protein. However, they caused changes in amino acid sequence which were proposed to disrupt a region of β-sheet secondary structure, which suggested that the mutations prevented recognition of UK 1 by *TuRB01* gene products. There were no obvious differences between wild-type and mutant cylindrical inclusion bodies, suggesting that the CI could still retain pathogenic function, but prevent elicitation of a defence response.

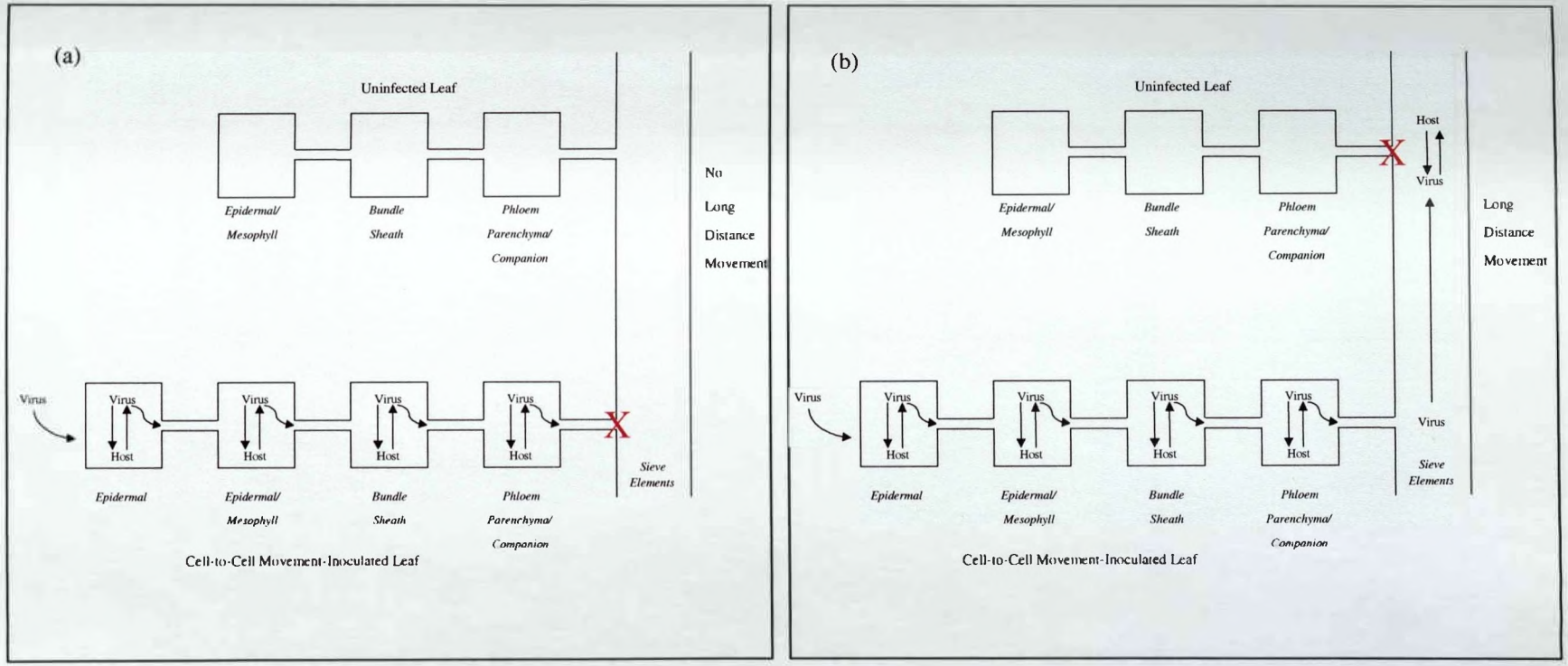


Figure 6.1 Possible mechanism of resistance models for detection of TuMV replication in inoculated leaves but not in uninoculated leaves. (a) Virus prevented from entering phloem sieve elements, hence no long distance movement. (b) Virus is able to move into phloem sieve elements but is unable to exit them into phloem parenchyma of uninfected leaves. X, virus cannot move any further than this point.

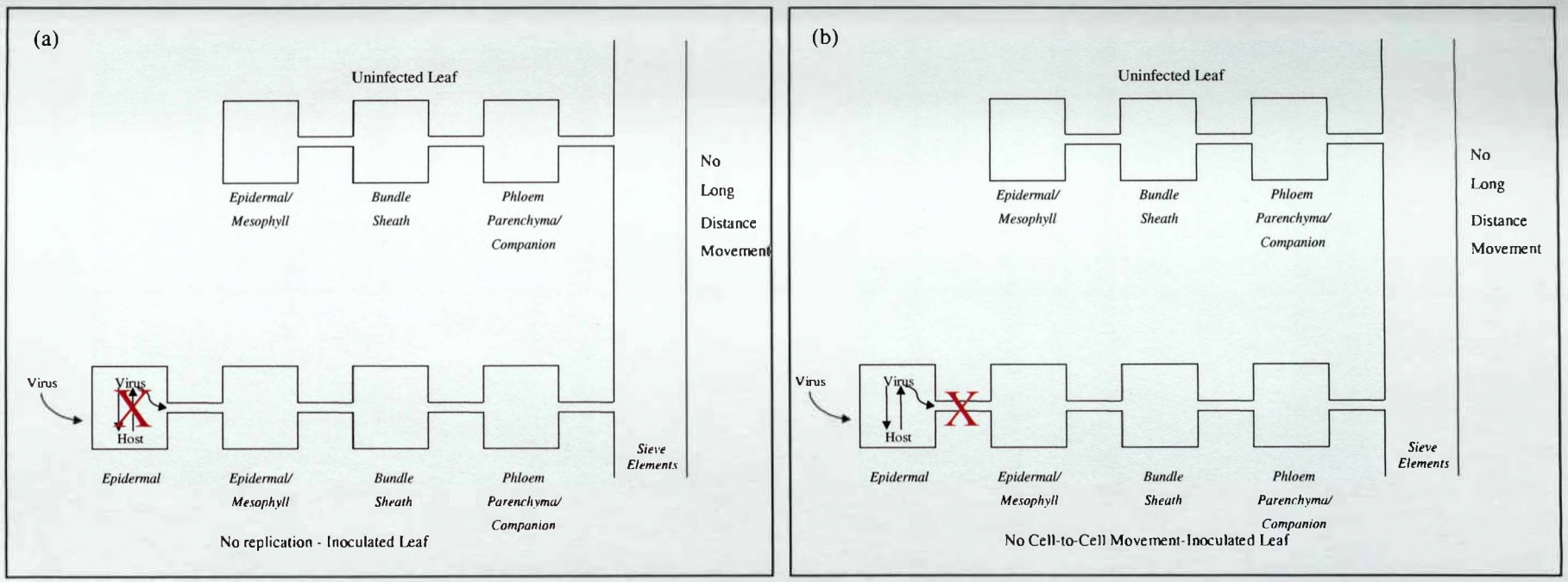


Figure 6.2 Model for possible mechanism of action for apparent immunity phenotype. (a) Virus replication is inhibited in infected cell. (b) Virus is able to replicate in infected cell, but cell-to-cell movement is either inhibited or limited. X, virus cannot move any further than this point.

The third aim was to characterise the genetic basis of TuMV resistance in some lines that were identified through the second aim. This was completed successfully using segregation ratios from screening backcross populations for six lines (*B. napus* lines 165 and 22S, *B. rapa* lines PI418957C, Jin G 55, BP058-4 and Jong Bai No. 2) and an F_2 population for *B. rapa* line Jong Bai No. 1. Models with single genes conferring dominant resistance were proposed for *B. rapa* line Jong Bai No. 2 and both *B. napus* lines, 165 and 22S. Models with single genes and multigenic models conferring recessive resistance were proposed for the rest of the *B. rapa* lines. In this study only simply inherited, dominant resistance has been identified in *B. napus*. This could be due to an evolutionary bottleneck that occurred during the formation of *B. napus* from the possible rare event of interspecies hybridisation that occurred between *B. rapa* and *B. oleracea*.

Another striking feature from this study is the frequency of recessive resistance in the *B. rapa* lines. Recessive traits are more likely to be expressed by self-compatible species, for example *B. napus*, yet the *B. rapa* lines were all self-incompatible. The resistances in the *B. rapa* lines may not be due to truly recessive alleles. Evidence from other studies suggested that resistance reported to result from homozygous recessive alleles could be explained by gene-dosage effects (Crute & Pink, 1996). In these cases the heterozygous host genotype was classed as susceptible even though pathogen growth was more restricted than it would have been in the homozygous susceptible genotype (Crute & Pink, 1996).

The recessive resistances in the majority of *B. rapa* lines tested and dominant resistance in the *B. napus* lines suggest that the resistance gene products operate in different ways. Fraser (1992) proposed that recessive resistance genes correspond to an alteration or deletion of a host factor that is required by the virus for movement or replication. Yang *et al.* (1997) suggested that products from dominant alleles act as recognition molecules that interact with a pathogen elicitor molecule, in a typical gene-for-gene type interaction, to induce a signal transduction defence pathway. The suggestion that some recessive alleles may also be involved in gene-for-gene type interactions (see Chapter 1) implies that

these gene products may also be involved in recognition of the virus. The complexity of some of the recessive resistance models, such as those proposed for *B. rapa* lines BP058-4 and Jong Bai No. 1, suggest that these genes may be involved in both recognition of the pathogen elicitor and also encode components of the signal transduction pathway, as described by Crute (1998).

The fourth aim was to identify molecular markers linked to TuMV resistance and to use these markers to poition the genes on a *B. napus* linkage map. The brassica lines with apparent single genes conferring dominant TuMV resistance, lines 22S, 165 and Jong Bai No. 2, were characterised using AFLP and/or microsatellite B-SA. The molecular analysis of line 22S confirmed the genetic model proposed with a single gene, *TuRB03*, being mapped to the lower region of linkage group N6 in the same region as *TuRB01* and *TuRB01b* (Walsh *et al.*, 1999; Rusholme, 2000), see Figure 5.14. The molecular analysis of *B. rapa* line Jong Bai No. 2 provided evidence for the validity of the genetic model proposed, with a single gene being positioned roughly at the upper region of linkage group N6 (Figure 5.14). Conversely the molecular analysis of *B. napus* line 165 did not confirm the single gene models proposed for dominant resistance to TuMV isolates UK 1 and CZE 1. The low frequency of polymorphisms identified through microsatellite B-SA, and the work being done on the avirulence determinants of UK 1 against 165 (C. Jenner, personal communication), suggest that the genetic control of this resistance is more complex. In fact, the two microsatellites that were apparently linked to TuMV resistance suggested that different genes were involved in resistance to UK 1, possibly on the upper region of linkage group N6, and CZE 1, possibly on N8 or N18 (Chapter 5).

The occurrence of the gene conferring dominant resistance in line 22S in the same region as *TuRB01* suggests these genes could be allelic. The F_1, F_2 and B_1 crosses for analysis of the genetic basis of TuMV resistance were produced using *B. napus* line N-o-1 as the susceptible parent. The resistance gene *TuRB01* confers UK 1 resistance in line N-o-1. The subsequent F_1 were all resistant to both UK 1 and CDN 1 (Table 4.4) and the individuals that were UK 1 resistant were CDN 1

resistant and those that were CDN 1 resistant were UK 1 resistant (data not shown). Unfortunately, time did not permit for the F_2 to be virus tested. Ideally the F_2 would have been tested with both CDN 1 and UK 1. If the CDN 1 resistant individuals were also resistant to UK 1 this would suggest that the genes are allelic and share some kind of common evolutionary background, which would support the idea of gene clustering seen for many other disease resistance genes. However, if the resistances to the TuMV isolates segregated independently of each other this would suggest that they were not allelic. If the genes were not allelic, but map in the same mapping interval it suggests that the map does not have enough resolution to separate them and further molecular work would have to be done to eventually clone both resistance genes and compare their nucleotide and peptide sequences.

I believe the most interesting source identified was the *B. napus* line 22S with novel resistance to the pathotype 4 isolate, CDN 1. As mentioned previously in Chapter 3, pathotype 4 isolates overcome all sources of resistance previously identified in *B. napus*. Line 22S was tested with more pathotype 4 isolates to find out if the resistance that is effective against the pathotype 4 type member, CDN 1, is also resistant against other pathotype 4 members. Line 22S was susceptible to all other pathotype 4 isolates tested, see Appendix 2 for data. This result suggests that CDN 1 is not typical of pathotype 4. To resolve this further, investigation into the avirulence determinant(s) of CDN 1 would have to be achieved. Unfortunately, it is very difficult to get the CDN 1 isolate to mutate to virulence on line 22S, with all attempts failing so far (C. Jenner, personal communication). Sequencing of CDN 1 and other pathotype 4 members, at the nucleotide and peptide levels, may provide some indication of possible reasons for CDN 1 inability to infect 22S.

Fraser (1992) suggested two models for resistance to viruses, a negative model and a positive model (see Chapter 1). The positive model for virus resistance, conferred by dominant resistance genes, is where the resistant host contains an inhibitor of the virus or develops one soon after infection (Fraser, 1992). The

virus is more likely to mutate to a virulent form in this model than in the negative model (Fraser, 1992). The narrow spectrum of resistance of *B. napus* line 22S suggests that it may be similar to the positive model described by Fraser (1992). There would be little benefit in using line 22S in breeding programmes because of its effectiveness against a narrow range of isolates. Line 22S could have other uses as an additional differential line to split isolates currently grouped into pathotypes 1-12. This line is now in use as a differential and has been used successfully to split pathotype 3 isolates into sub-groups (P.J. Hunter, personal communication).

FUTURE WORK

The markers identified in this work could be used in future work to identify collinear regions in *A. thaliana*. This would assist in fine mapping to identify markers tightly linked to TuMV resistance. Tightly linked markers could be used for MAS to deploy the resistance genes through breeding programmes. They would also facilitate map-based cloning of the resistance genes. Map-based cloning would be easier in *A. thaliana* than *Brassica* spp., because of the wide number of resources available for *A. thaliana*. Cloned resistance genes could then be rapidly deployed through the genetic transformation of TuMV-susceptible brassica cultivars. The most efficient way to deploy virus resistance genes is to pyramid genes through either marker assisted selection to combine large numbers of genes or alternatively deploy the resistance genes through multilines. Multilines are where the individuals of the crop are essentially the same as each other, but are different at specific loci. If different individuals had combinations of different resistance genes the virus would be less likely to mutate to overcome resistance.

Cloning of the TuMV resistance genes will also allow the elucidation of function of resistance gene products. TuMV is able to mutate relatively easily to overcome resistance (C. Jenner, personal communication). To deploy resistance genes effectively to avoid the "boom-bust" cycle (Crute & Pink , 1996; Pink &

Puddephat, 1999) it is also important to develop knowledge about the virus. The development of infectious clones, expressing green-fluorescent protein (GFP) or β-glucuronidase reporter gene (GUS), and yeast-two hybrid systems will allow more detailed information to be obtained about the way virus products interact with resistance gene products. Hence information about the different mechanisms of resistance and apparent immunity may be obtained.

The identification of markers tightly linked to TuMV resistance will also allow the introgression of resistance from *B. rapa* and *B. napus* into *B. oleracea.* Attempts are currently underway to transfer TuMV resistance from *B. rapa* to *B. oleracea* (R. Rusholme, 2000).

Markers tightly linked to TuMV resistance will also allow the introgression of separate resistance alleles into susceptible inbred/doubled haploid backgrounds, so the individuals would be identical apart from resistance loci. Subsequent inoculation of these lines with a range of TuMV isolates would provide information about the specificity of each resistance gene. These lines could also be used for allelism tests, as well as finding out about the interaction of the different genes, by crossing the inbred/doubled haploid lines containing different resistance genes.

APPENDIX 1

GENETIC MODELS

Genotypes of F_1 gametes	Genotype of susceptible parent gamete
	r
R	Rr *Resistant*
r	rr Susceptible

Expected Segregation Ratio *1 Resistant:1 Susceptible*

Figure A1 Punnett Square of backcross generation genotypes and phenotypes produced when an F_1 and susceptible parent are crossed when dominant alleles at a single locus are required for turnip mosaic virus resistance. R, dominant resistance allele; r, recessive susceptibility allele. Expected phenotypic segregation ratio is shown below the Punnett Square.

Genotypes of F_1 gametes	Genotypes of resistant parent gametes
	s
s	ss *Resistant*
S	Ss Susceptible

Expected Segregation Ratio *1 Resistant:1 Susceptible*

Figure A2 Punnett Square of backcross generation genotypes and phenotypes produced when an F_1 and resistant parent are crossed when recessive alleles at a single locus are required for resistance to turnip mosaic virus isolate. S, dominant susceptibility allele; s, recessive resistance allele. Expected phenotypic segregation ratio is shown below the Punnett Square.

Genotype of F_1 gametes	Genotype of resistant parent gametes
	s_1s_2
S_1S_2	$S_1s_1S_2s_2$
	Susceptible
S_1s_2	$S_1s_1s_2s_2$
	Resistant
s_1S_2	$s_1s_1S_2s_2$
	Resistant
s_1s_2	$s_1s_1s_2s_2$
	Resistant

Expected Segregation Ratio: *3 Resistant:1 Susceptible*

Figure A3 Punnett Square of backcross generation genotypes and phenotypes produced when an F_1 and resistant parent are crossed when homozygous recessive alleles at either of two epistatic loci are required for resistance to turnip mosaic virus. S_1 and S_2, dominant susceptibility alleles; s_1 and s_2, recessive resistance alleles. Expected phenotypic segregation ratio is shown below the Punnett Square.

Genotype of F_1 gametes	Genotype of resistant parent gametes s_3s_4
S_4S_5	$S_3s_3S_4s_4$
	Susceptible
S_3s_4	$S_3s_3s_4s_4$
	Susceptible
s_3S_4	$s_3s_3S_4s_4$
	Susceptible
s_3s_4	$s_3s_3s_4s_4$
	Resistant

Expected Segregation Ratio: *1 Resistant:3 Susceptible*

Figure A4 Punnett Square of backcross generation genotypes and phenotypes produced when an F_1 and resistant parent are crossed when homozygous recessive alleles at two complementary loci are required for resistance to turnip mosaic virus. S_3 and S_4, dominant susceptibility alleles; s_3 and s_4, recessive resistance alleles. Expected phenotypic segregation ratio is shown below the Punnett Square.

Genotypes of gametes	Genotypes of gametes							
	$S_5S_6S_7$	$s_5S_6S_7$	$S_5s_6S_7$	$S_5S_6s_7$	$s_5s_6S_7$	$s_5S_6s_7$	$S_5s_6s_7$	$s_5s_6s_7$
$S_5S_6S_7$	$S_5S_5S_6S_6S_7S_7$ Susceptible	$S_5s_5S_6S_6S_7S_7$ Susceptible	$S_5S_5S_6s_6S_7S_7$ Susceptible	$S_5S_5S_6S_6S_7s_7$ Susceptible	$S_5s_5S_6s_6S_7S_7$ Susceptible	$S_5s_5S_6S_6S_7s_7$ Susceptible	$S_5S_5S_6s_6S_7s_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible
$s_5S_6S_7$	$S_5s_5S_6S_6S_7S_7$ Susceptible	$s_5s_5S_6S_6S_7S_7$ Susceptible	$S_5s_5S_6s_6S_7S_7$ Susceptible	$S_5s_5S_6S_6S_7s_7$ Susceptible	$s_5s_5S_6s_6S_7S_7$ Susceptible	$s_5s_5S_6S_6S_7s_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible	$s_5s_5S_6s_6S_7s_7$ Susceptible
$S_5s_6S_7$	$S_5S_5S_6s_6S_7S_7$ Susceptible	$S_5s_5S_6s_6S_6S_7S_7$ Susceptible	$S_5S_5s_6s_6S_7S_7$ Susceptible	$S_5S_5S_6s_6S_7s_7$ Susceptible	$S_5s_5s_6s_6S_7S_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible	$S_5S_5s_6s_6S_7s_7$ Susceptible	$S_5s_5s_6s_6S_7s_7$ Susceptible
$S_5S_6s_7$	$S_5S_5S_6S_6s_7s_7$ Susceptible	$S_5s_5S_6S_6S_7S_7$ Susceptible	$S_5S_5S_6s_6S_7s_7$ Susceptible	$S_5S_5S_6S_6s_7s_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible	$S_5s_5S_6S_6S_7s_7$ Susceptible	$S_5S_5S_6s_6S_7s_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible
$s_5s_6S_7$	$S_5s_5S_6s_6S_7S_7$ Susceptible	$s_5s_5S_6s_6S_7S_7$ Susceptible	$S_5s_5s_6s_6S_7S_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible	***$s_5s_5s_6s_6S_7S_7$*** ***Resistant***	$s_5s_5S_6s_6S_7s_7$ Susceptible	$S_5s_5s_6s_6S_7s_7$ Susceptible	***$s_5s_5s_6s_6S_7s_7$*** ***Resistant***
$s_5S_6s_7$	$S_5s_5S_6S_6S_7s_7$ Susceptible	$s_5s_5S_6S_6S_7s_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible	$S_5s_5S_6S_6s_7s_7$ Susceptible	$s_5s_5S_6s_6S_7s_7$ Susceptible	***$s_5s_5S_6S_6s_7s_7$*** ***Resistant***	$S_5s_5S_6s_6S_7s_7$ Susceptible	***$s_5s_5S_6s_6s_7s_7$*** ***Resistant***
$S_5s_6s_7$	$S_5S_5S_6s_6S_7s_7$ Susceptible	$S_5s_5S_6S_6S_7s_7$ Susceptible	$S_5S_5s_6s_6S_7s_7$ Susceptible	$S_5S_5S_6s_6S_7s_7$ Susceptible	$S_5s_5s_6s_6S_7s_7$ Susceptible	$S_5s_5S_6s_6S_7s_7$ Susceptible	***$S_5s_5s_6s_6s_7s_7$*** ***Resistant***	***$S_5s_5s_6s_6s_7s_7$*** ***Resistant***
$s_5s_6s_7$	$S_5s_5S_6s_6S_7s_7$ Susceptible	$s_5s_5S_6s_6S_7s_7$ Susceptible	$S_5s_5s_6s_6S_7s_7$ Susceptible	$S_5s_5S_6s_6s_7s_7$ Susceptible	***$s_5s_5s_6s_6S_7s_7$*** ***Resistant***	***$s_5s_5S_6s_6s_7s_7$*** ***Resistant***	***$S_5s_5s_6s_6s_7s_7$*** ***Resistant***	***$s_5s_5s_6s_6s_7s_7$*** ***Resistant***

Expected Segregation Ratio *10 Resistant:54 Susceptible*

Figure A5 Punnett Square of F_2 generation genotypes and phenotypes produced when an F_1 is selfed when homozygous recessive alleles at three complementary loci where any two are epistatic over the third for resistance to turnip mosaic virus. S_5, S_6 and S_7, dominant susceptibility alleles; s_5, s_6 and s_7, recessive resistance alleles. Expected phenotypic segregation ratio is shown below the Punnett Square.

APPENDIX 2

FURTHER CHARACTERISATION OF *B. NAPUS* LINE 22S

MATERIALS AND METHODS

Plant Material

Selfed progeny of line 22S were grown and maintained as described in Chapter 3, Section 3.3.1.

Virus Isolates

Virus isolates used in this experiment are listed in Table A. They were selected because they are from a range of areas throughout the world and are members of pathotype 4. CHN12 x165 was chosen because it is similar to Pathotype 4 but produces a necrotic reaction on differential line 165 (J. A. Walsh, personal communication). All isolates were maintained as described in Chapter 3, Section 3.3.2.

Inoculation procedure

As described in Chapter 3, Section 3.3.3.

Assessment of infection

As described in Chapter 3, Section 3.3.4.

RESULTS

Table A **Results of testing 22S self progeny with turnip mosaic virus isolates from pathotype 4 and isolate CHN 12 x165 that is 'pathotype 4-like.'**

Virus Isolate (Pathotype)	Phenotype of 22S self progeny
GBR 6 (4)	14/14 +
POL 1 (4)	15/15 +
CZE 20 (4)	19/19 +
DEU 2 (4)	16/16 +
HUN 1 (4)	16/16 +
DEU 9 (4)	20/20 +
CHN 12 x165 (pathotype 4-like)	19/19 $+_N$

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