The Location of *Tu* on the Genetic Map of *Lactuca sativa* and the Identification of Random Amplified Polymorphic DNA Markers Flanking and Tightly Linked to *Tu*

by

©Marjorie Robbins

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

Department of Plant Science
Macdonald Campus of McGill University
Montréal, Québec

July, 1993

Suggested short title:

RAPD markers linked to turnip mosaic virus resistance in lettuce

Abstract

In *Lactuca sativa*, the dominant gene *Tu* confers resistance to infection by turnip mosaic virus (TuMV). *Tu* and *Dm*5/8, a gene for resistance to *Bremia lactucae*, are linked in *L. sativa*. The area surrounding *Dm*5/8 on the genetic map of *L. sativa* contains restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers. The orientation of *Tu* relative to *Dm*5/8 was not known. Locating *Tu* would indicate which markers are on the map of lettuce close to *Tu*. To locate *Tu* on the *L. sativa* genetic map, F_3 families from recombinant F_2 in the *Dm*5/8 area of a cross between TuMV-resistant (Cobbham Green) and susceptible (Calmar) cultivars were inoculated with TuMV and phenotyped for *Tu* by indirect enzyme-linked immunosorbent assay. Polyclonal antibodies for immunodetection were produced using turnip mosaic virus coat protein expressed in *E. coli*. Phenotypic ratios within F_3 families were used to determine individual F_2 genotypes for *Tu*. With these genotypes, *Tu* was located on the genetic map of *L. sativa* relative to data present for *Dm*5/8 and surrounding markers, between OPM18 and OPY13. Using bulked segregant analysis, bulks created for the *Dm*5/8 locus were screened for genetic polymorphisms by the RAPD technique. Five new RAPD markers, *UBC346*, *UBC517*, *UBC563*, *UBC599*, and *UBC675* were found linked to *Tu* after mapping relative to F_2 genotypes for *Tu* and other RAPD markers. The resulting three-point mapping information indicates that *Tu* is flanked by two markers, *OPM18/OPL08* and *UBC346*, at respective genetic distances of 0.4 and 0.7 cM.

Résumé

Parmi les plantes appartenant à l'espèce *Lactuca sativa*, le gène dominant *Tu* est suffisant pour conférer une résistance à l'infection par le virus de la mosaïque du navet (TuMV). *Tu* et *Dm*5/8, un gène permettant la résistance au *Bremia lactucae*, sont liés dans *L. sativa*. La région entourant *Dm*5/8 sur la carte génétique de la laitue contient beaucoup de marqueurs de polymorphismes de longueur de fragments de restriction (RFLP) et d'ADN polymorphique amplifié (RAPD). L'orientation de *Tu* par rapport à *Dm*5/8 n'était pas connue. La localisation de *Tu* permettrait d'identifier les marqueurs rapprochés de celui-ci sur la carte génétique de la laitue. Pour ce faire, une population F_3 issu d'un croisement entre un cultivar résistant au TuMV (Cobbham Green) et un cultivar susceptible au TuMV (Calmar) fut inoculée avec du TuMV. Les phénotypes de chaque plantes furent déterminés par analyse ELISA. Des anticorps polyclonaux pour l'immunodétection ont été produits en utilisant une protéine recombinante de la capside du TuMV exprimée dans *E. coli*. Le rapport phénotypique dans la population F_3 a été utilisé pour déterminer les génotypes de la population F_2 concernant *Tu*. Avec ces génotypes, *Tu* a été localisé sur la carte génétique de *L. sativa* dans la région où se trouvait *Dm*5/8, entre *OPM18* and *OPY13*. En utilisant l'analyse de ségrégation de groupes, des groupes créés pour le locus *Dm*5/8 ont été examinés pour la présence de polymorphismes génétiques par la technique de RAPD. Cinq nouveau marqueurs RAPD, *UBC346*, *UBC517*, *UBC563*, *UBC599*, et *UBC675* ont été liés à *Tu* après la cartographie relative aux génotypes de la population F_2 pour *Tu* et d'autres marqueurs RAPD. La cartographie à trois points indique que *Tu* est entouré de deux marqueurs, *OPM18/OPL08* et *UBC346*, à une distance génétique respective de 0.4 et 0.7 cM.

Acknowledgements

I am indebted to my supervisor, Dr. M.G. Fortin, for his guidance on a very interesting project for the past two years. His financial support from a grant from the Natural Sciences and Engineering Research Council was greatly appreciated. I would like to thank Dr. J. Peterson and Dr. S. Sparace for being on my research committee. I am grateful to Dr. J. Peterson for guidance relative to virus morphology and transmission electron microscopy. Much thanks to Mr. G. Rimmer and Mrs. H. Cohen-Rimmer for electron microscopy and photographic assistance respectively. I wish to express my gratitude to Dr. J.-F. Laliberté for providing the pET-CP expression system and Dr. R.W. Michelmore for recombinant seed necessary for this project. I am grateful for the assistance of Mr. N. Urbani in the radioactive probing and for Mr. N. Tinker's helpful suggestions on everything along the way. I would like to thank Eliane Ubalijoro (for translating the abstract) and Nicola for walking me home late at night and putting up with my "Just one more hour..." and for their friendship. Finally, I cannot forget Andrew, my unpaid technician, for waiting patiently these two years before going on with his own life.

Table of Contents

List of Figures

List of Tables

I. Introduction

Plant responses to viral infection are determined in many cultivated crops by single resistance genes (Matthews, 1991; Fraser, 1990; Ellis *et al.*, 1988). Several models have been proposed to account for the inhibition of viral multiplication in resistant plants by resistance gene products, but in the absence of a cloned candidate gene, the causal relationship to the resistance phenotype has been difficult to confirm (Fraser, 1987; Bruening *et al.*, 1987; Ponz *et al.*, 1988).

Several proteins, enzymes and mRNAs are induced or repressed upon viral infection (Legrand *et al.*, 1987; Kombrink *et al.*, 1988). Many of these factors can also be induced by abiotic stresses, making their role in resistance difficult to establish (Bowles, 1990). Differentially expressed genes are thought to be involved in the general stress defense reaction mounted by the plant cell in response to infection, and are not the determinants of the resistance phenotype. In contrast, plant viral resistance genes expressed either constitutively or in a regulated manner are specific for one or a few strains of a virus (Fraser, 1987), and are the key genetic factor that determines whether the plant will be resistant or susceptible.

The use of molecular markers to determine map location followed by map-based cloning is a promising approach for the isolation of viral resistance genes from plants. Human disease genes such as the cystic fibrosis gene have been cloned using map-based cloning (Rommens *et al.*, 1989). No viral resistance gene has yet been cloned from plants.

A single dominant gene, *Tu*, confers resistance to turnip mosaic virus (TuMV) in *L. sativa* (Zink and Duffus, 1970). TuMV represents a significant problem in the *Brassicaceae*, particularly in economically important crops such as rutabaga (*Brassica. napus* subsp. *napobrassica*) and canola (*B. napus* subsp. *oleifera*) (Shattuck and Stobbs, 1987). No complete resistance to TuMV has yet been found in the *Brassicaceae* (Doucet *et al.*, 1990; Pink and Walkey, 1990).

A detailed genetic linkage map for *L. sativa* (Kesseli *et al.*, unpublished; Kesseli *et al.*, 1991) has been prepared using Restriction Fragment Length Polymorphism (RFLP) (Beckman and Soller, 1983) and Random Amplified Polymorphic DNA markers (RAPD) (Williams *et al.*, 1990). Yeast artificial chromosomes (YACs) of up to one million base pairs have been reported. A partial YAC library containing lettuce genomic DNA (genome size of *L. sativa* is 2.6×10^9 bp, Arumuganathan and Earle, 1991) has been produced (Fortin *et al.*, unpublished) which could be used for genomic walking experiments. Markers in the area of *Tu* may be useful in screening these YACs.

In screening for RFLP or RAPD markers, few polymorphisms between cultivars segregate with the target gene. Cultivars Calmar (a crisphead lettuce) and Cobbham Green (a butterhead), while differing at the *Tu* locus, are polymorphic at many other loci. Most markers detected between these two cultivars will not be linked to *Tu*. Near isogenic lines (NILs) increase the likelihood of polymorphisms being linked to a gene of interest. For example, 50% of the polymorphisms between NILs of lettuce produced after five backcrosses were linked to the target locus (Paran *et al.*, 1991). Two cultivars which differed at the *Dm*11 locus were used to produce these NILs. The percent of

polymorphisms linked to a target locus using NILs varies with cultivar relatedness and the number of backcrosses performed. In bulked segregant analysis, markers linked to the target region are screened for using pools of DNA from resistant and susceptible homozygous individuals originating from a single cross (Michelmore *et al.*, 1991). All polymorphic markers which identify a sequence divergence between two pools of adequate size (i.e. 10 or more individuals per bulk) are linked to the target locus.

The first step towards cloning and characterization of the *Tu* resistance gene is the identification of closely linked and flanking molecular markers. As the genomic region surrounding *Dm*5/8 contains many RFLP and RAPD markers (Kesseli *et al.*, unpublished; Kesseli *et al.*, 1991); it is feasible that situating *Tu* on the genetic map of lettuce will reveal some markers which are tightly linked to *Tu*.

Objectives

1. To produce an antibody to turnip mosaic virus coat protein (TuMV-CP) using recombinant TuMV-CP expressed in *Escherichia coli*.

An enzyme-linked immunosorbent assay (ELISA) was used to score individuals as either resistant or susceptible to TuMV-infection. The genotyping experiment for the location of *Tu* utilized enzyme-linked immunosorbent assay (ELISA) (see objective 2). Anti-TuMV antibodies for ELISA were not commercially available. The TuMV-CP was cloned into an expression vector (pET-CP) by J.-F. Laliberté. pET-CP was obtained and antibodies to TuMV-CP produced by injecting rabbits with TuMV-CP expressed in *E.*

coli.

2. To locate *Tu* relative to *Dm*5/8 on the genetic map of *Lactuca sativa*.

A population segregating for *Dm*5/8 was examined for recombinants in the area of *Dm*5/8 by R.W. Michelmore (University of California, Davis). As *Tu* is close to *Dm*5/8, the genetic information for recombinant individuals in the area of *Dm*5/8 was used to locate *Tu*. F_3 families derived from F_2 recombinant individuals were provided by R. W. Michelmore. These individuals were phenotyped for resistance or susceptibility to TuMV by ELISA. Phenotypic ratios within F_3 families determined F_2 genotypes at *Tu*. Incorporating the genotypes for *Tu* with the mapping information in the area of *Dm*5/8 provided by R.W. Michelmore gave the location of *Tu* on the genetic map of *L. sativa*.

3. To find molecular markers flanking and tightly linked to *Tu* using bulked segregant analysis.

Many markers in the genetic area surrounding *Dm*5/8 have been mapped by R. W. Michelmore. By situating *Tu* on this map, some markers may be closely linked to *Tu*. Two pools of DNA (bulks) for the *Dm*5/8 locus produced by R. W. Michelmore were screened for polymorphism. Polymorphisms between pools were examined for cosegregation with the *Tu* locus. Genotypes for new RAPD loci were added to the mapping information provided by R. W. Michelmore and to the genotypes for *Tu* (see objective 2) to produce a genetic map of the area surrounding *Tu*.

II. Literature Review

1. Mechanisms of resistance to plant viruses

Monogenic resistance to viruses in cultivated crops is conferred in many cases by single genes (Fraser, 1987; Ellis *et al.*, 1988; Matthews, 1991). At least twenty-nine dominant viral resistance genes are known in plants (Fraser, 1987). Recessive viral resistance genes are not uncommon, both types of gene often occurring for different viruses in the same plant species (van Loon, 1987). In *L. sativa*, a dominant and a recessive gene confer resistance to turnip mosaic virus (TuMV) and lettuce mosaic virus (LMV) respectively (Zink and Duffus, 1970; Walkey *et al.*, 1985).

Plant viral resistance genes, such as the dominant *I* gene in *Phaseolus* beans against bean common mosaic virus (BCMV), confer long-term resistance, in this case lasting at least 40 years (Walkey, 1985). Virulent isolates of BCMV overcoming the *I* gene have been reported but have not become prevalent in the field (Walkey, 1985). Resistance to potato virus X in potato cultivars such as Epicure and King Edward in Britain has lasted more than 50 years (Walkey, 1985).

The mechanisms by which resistance genes prevent viral infection remain unclear. Two theories that explain the interaction between viruses and viral resistance genes in plants are the gene-for-gene theory of Flor (1971) and the positive/negative model proposed by Fraser (1987).

1.1 The gene-for-gene theory

In the gene-for-gene theory, each allele in the host that confers resistance is complementary to a virulence locus in the pathogen which can overcome resistance (Flor, 1971). Complementation between a plant resistance gene and an avirulence gene in the pathogen causes pathogen localization observed in the plant as a hypersensitive response (HR). In HR, localized cell death at the site of infection prevents systemic spread of the pathogen.

The limited size of viral genomes makes it unlikely that a viral gene would have as its sole function avirulence or virulence (Fraser, 1990; Padgett and Beachy, 1993). Fraser (1983) observed that differences in virulence between strains of TMV was not due to their ability to replicate in the host but was correlated with differences in the thermal stabilities of their coat protein subunits. It was demonstrated by Saito *et al.* (1987) that a factor coded for by the TMV-L (tomato strain) coat protein gene was responsible for the hypersensitivity response of *Nicotiana sylvestris* containing the *N'* gene. Tobacco carrying the *N'* gene was susceptible to TMV-OM (common strain), causing systemic spread and mosaic symptoms upon infection. Knorr and Dawson (1988) observed that a point mutation of TMV-OM (common strain) caused a single amino acid change in the viral coat protein. This mutation caused a HR to the virus in tobacco instead of the usual systemic infection (Knorr and Dawson, 1988).

Proteins other than viral CP have produced changes in the interaction between virus and host following mutation. No mutation in the CP is known to induce the *N* gene-mediated HR by tobamoviruses (reviewed by Culver *et al.*, 1991). Mutations in

the 126 kDa protein of a tomato mosaic virus (ToMV) strain overcame the resistance conferred by the *Tm-1* gene in tomato (Meshi *et al.*, 1988). Mutations in strains of ToMV which overcame the *Tm-2* and *Tm-2*^{2} resistance genes were found to have occurred in the movement protein (MP) gene (Meshi *et al.*, 1989; Calder and Palukaitis, 1992). From cloning and sequencing of a mutant tobamovirus, Ob, which could overcome the *N* gene-mediated HR and produce systemic infection in tobacco carrying the *N* gene, Padgett and Beachy (1993) found that the Ob MP alone did not overcome the *N* gene-mediated response. Mutagenesis of Ob which reversed the response, as observed by a HR on tobacco, was found to have occurred as a single nucleotide change in the 126 kDa gene (Padgett and Beachy, 1993).

Molecular studies pertaining to the gene-for-gene theory are facilitated by the small genome size of plant viruses and their relative ease of cloning. Examination of how viral genes interact with host resistance genes will be furthered by the cloning of viral resistance genes from plants.

To date, the gene-for-gene theory has been more closely examined on the level of the virus than of the host. The positive/negative model of viral resistance proposed by Fraser (1987) examines the mechanisms of resistance conferred by host genes.

1.2 A positive and a negative model of resistance

A positive and a negative model for viral resistance which predict plant responses to infection has been proposed based on data from the interactions between different viruses and their hosts (Fraser, 1987). The negative model explains the interaction

between recessive host genes and viruses, where complete immunity is conferred onto a resistant plant by the absence of a gene product (Fraser, 1987). In the positive model, a resistant plant produces an inhibitor of viral replication (Fraser, 1987). Plants with dominant genes displaying a positive mechanism of resistance recognize viral invasion and "switch on" resistance (Fraser, 1987).

From an examination of host-virus combinations, Fraser (1987) suggests that virus localization, as evidenced by a HR to infection, is associated with the presence of dominant genes. The majority of dominant viral resistance genes examined to date fit Fraser's (1987) positive model (Fraser, 1990).

Some dominant alleles confer complete immunity to viral infection (Fraser, 1990). Complete immunity conferred by a single locus is defined by the absence of symptoms and no HR response. Such cases may not fit Fraser's (1987) positive model as, to produce a rapid response to viral invasion and prevent HR, the resistance gene must produce its putative product constitutively. Such a mechanism may be present in the cowpea cv. Arlington where a protease inhibitor has been shown to inhibit polyprotein processing of cowpea mosaic virus (CPMV) (Ponz *et al.*, 1988). Resistance to CPMV in Arlington cowpeas is conferred by a dominant gene whose putative product may be expressed constitutively (Bruening *et al.*, 1987). A direct genetic relationship between this constitutively expressed protein and the dominant resistance gene has yet to be determined.

Fraser's (1987) theory does not fit all interactions between viruses and their hosts, as seen in the cowpea example above. One method of examining the role of plant genes

in the resistance phenotype would be the cloning of such genetic elements and examination of their putative products for function during the resistance response.

2. The link between resistance gene and resistance mechanism

Cosegregation of a genetic locus with resistance indicates that it is the cause of that resistance phenotype. Specific resistance conferred by such genes is distinct from non-host resistance (Fraser, 1982). Non-host resistance results from incompatibility between host and pathogen and does not segregate as a single genetic locus, but instead is controlled by a multitude of loci (Fraser, 1982; 1987). Cross-protection produced by transformation of susceptible cultivars with cloned viruses or viral elements is also considered as separate from specific genetic immunity as such elements produce a reduction of symptom severity and a delay in symptom appearance which is not sufficient to produce complete immunity (Harrison, *et al.*, 1987; Abel *et al.*, 1986; reviewed by White and Antoniw, 1991; Wilson, 1993). Only specific genetic resistance which is complete and heritable as a single locus will be examined further.

Ponz *et al.* (1988), in cellular studies with protoplasts (plant cells which have been separated from each other by degradation of the cell wall), correlated the presence of an inhibitor of polyprotein processing with resistance to CPMV. Isolation and characterization of this inhibitor and segregation studies using crosses of resistant and susceptible cowpea lines have yet to be done.

To complement cellular studies of resistance, various strategies have been used to attempt to isolate the genetic sequences responsible for resistance, either indirectly

through protein products (section II.2.1) or directly by the establishment of genetic maps necessary for map-based cloning of genes (section II.2.2).

2.1 Proteins induced by infection

Pathogenesis-related (PR) proteins were first found in tobacco cultivars displaying hypersensitivity to TMV (Gianinazzi *et al.*, 1970). Gianinazzi *et al.* (1977) later examined four protein components called 'b' proteins which were found in TMV-infected tobacco leaves. PR proteins account for the major quantitative changes in soluble host-encoded proteins in infected plants as compared to healthy plants (Pierpoint, 1983; 1986). Such proteins have now been described in at least 20 plant species after infection with pathogens or treatment with chemicals (reviewed by Redolfi, 1983; van Loon, 1985; Bol, 1988; Carr and Klessig, 1989).

Induced resistance displayed as a HR has been attributed to the presence of PR proteins and an inhibitor of virus replication (IVR) (Loebenstein and Gera, 1981, Spiegel *et al.*, 1989). Compounds such as polyacrylic acid (Gianinazzi and Kassanis, 1974) and aspirin (White, 1979) have induced production of PR proteins without a HR. None of the PR proteins or IVR has yet been shown to be the direct product of a viral resistance gene. It is now thought that PR proteins are involved in host responses to invading organisms or some form of stress, a theory held up by the wide diversity of their modes of action, ranging from inhibitors and enzymes to structural wall proteins (Bowles, 1990).

2.2 Map-based cloning of resistance genes

Correlation between protein and resistance phenotype in plants has remained elusive. One approach which has been employed successfully for the isolation of human disease genes where the gene product is not known is map-based or positional cloning (Rommens *et al.*, 1989). No viral resistance gene has yet been cloned from plants.

The first step towards cloning a target gene is the identification of closely linked markers flanking the target locus, followed by the estimation of the physical distance between markers (Wicking and Williamson, 1991). After the discovery of markers physically flanking the locus, chromosome walking and jumping were used to narrow the region between the markers in the search for candidate genes for the cystic fibrosis phenotype (Rommens *et al.*, 1989).

Both RFLP and RAPD markers have recently been found by recombinantion frequency analysis to be linked to resistance genes in plants (Gebhart *et al.*, 1993; Martin *et al.*, 1993; Timmerman *et al.*, 1993), none of which have been reported to be physically tightly linked to the loci of interest. Markers are tightly linked when the physical distance (in base-pairs) separating them allows chromosome walking. The physical distance between markers is determined using pulsed-field gel electrophoresis (PFGE). By alternating the angle of the current being applied in gel electrophoresis, large DNA fragments are resolved. This strategy was necessary for the location of the cystic fibrosis gene where clones were oriented relative to each other on the chromosome using rare-cutting restriction enzymes and PFGE (Rommens *et al.*, 1989).

No molecular markers located within a walkable distance have yet been reported

for a plant viral resistance gene. Timmerman *et al.* (1993) have recently found RFLP markers linked to the pea seed-borne mosaic virus resistance locus, *sbm-1*, in pea. The closest marker is 8 cM away from the resistance locus.

RFLP markers were found closely linked by recombination frequency to *Tm*-2a, a gene conferring resistance to TMV in tomato (Young *et al.*, 1988). However, the physical base-pair equivalent of 1 cM in the area of *Tm*-2a was 4 Mb, much greater than the average of 500 kb per centiMorgan in tomato (Ganal *et al.*, 1989). The genetic distance of 0.4 cM is equivalent to 2 Mb on the physical map of this area of the genome. This greater physical distance is due to the proximity of *Tm*-2a to the centromere, where recombination is suppressed by heterochromatin (Tanksley *et al.*, 1992). Large populations of tomato must be screened for recombinants to obtain a higher resolution of markers close to *Tm*-2a.

Molecular markers tightly linked to target loci are necessary for map-based cloning. Three major types of molecular markers, isoenzymes, RFLPs, and RAPDs, can be identified for genomic regions of interest.

2.2.1 Types of genetic markers

(i) Isoenzymes

The isozyme technique relies on the existence of different isoforms of various enzymes. These isoforms migrate differentially on starch gels and can be examined for polymorphism between individuals. Such polymorphisms are usually codominant and show no epistatic effects as do some morphological markers (Tanksley, 1983).

By focusing on differences at the protein level, the isozyme technique relies on the examination of differences in expressed regions. Expressed regions of plant genomes comprise 5% of the nuclear DNA (Singer and Berg, 1991). Therefore, 95% of the nuclear DNA is unavailable for analysis using isoenzymes as genetic markers.

(ii) Restriction fragment length polymorphisms (RFLP)

The use of restriction endonucleases which selectively cleave DNA, and techniqes to transfer DNA to solid supports and to detect low copies of homologous DNA, have enabled the direct mapping by RFLP of previously inaccessible (or unexpressed or non-coding) genetic loci in tandem with phenotypic markers (Zabeau and Roberts, 1979).

Molecular mapping of higher eukaryotes began with the construction of a human genetic linkage map using RFLPs (Botstein *et al.*, 1980, reviewed by Tanksley *et al.*, 1989). RFLP markers are often codominant, display two or more allelic forms and show no pleiotropic effects, making them ideal for the production of genetic maps (Beckmann and Soller, 1983). RFLPs are identified by probing genomic DNA cut with restriction endonucleases and mapping the resulting alleles for segregation with genes of interest.

RFLPs have been used extensively in recent years to map economically important plant genomes such as corn (Coe *et al.*, 1990), tomato (Tanksley and Mutscher, 1990) and lettuce (Landry *et al.*, 1987).

(ii) Random amplified polymorphic DNA (RAPD)

Polymerase chain reaction (PCR) technology offers alternatives to RFLP for

generating molecular markers (Saiki *et al.*, 1985, Saiki *et al.*, 1988). PCR involves the use of a thermostable DNA polymerase, nucleotide triphosphates (dNTP), a reaction buffer containing $MgCl_2$ necessary for polymerase function, oligonucleotide primers, and a DNA template.

One PCR-based technique for the identification of genetic markers involves the use of 10-base oligonucleotide primers to generate RAPDs (Williams *et al.*, 1990). RAPD primers are designed to have a minimum GC content of 50% to facilitate binding of primer to DNA template (Williams *et al.*, 1990). RAPDs do not require any specific sequence information from the target genome as is required by other PCR-based techniques.

Paran *et al.* (1991) showed that, in identifying RAPD markers linked to three loci for downy mildew resistance in lettuce, RAPDs were greater than 10-fold more efficient in terms of manpower and costs than screening for the same polymorphisms using RFLP technology. Genomic and cDNA probes which fail to detect polymorphism contribute to the lower efficiency of RFLP in this case (Paran *et al.*, 1991).

Paran *et al.* (1991) found a contrast in the distribution of RFLP and RAPD markers in the downy mildew resistance gene (*Dm*) regions of *L. sativa*. No RFLPs were detected linked to *Dm*11 using near isogenic lines (NILs) produced for *Dm*11 while seven RAPDs were mapped to a 34 cM region surrounding the *Dm*11 locus (Paran *et al.*, 1991). The absence of RFLP in this area may reflect a lack of expressed genes or genes which are expressed at low levels, having lower representation in the cDNA library (Paran *et al.*, 1991). RAPDs may contain repetitive DNA, while RFLPs are selected to

contain low copy sequences. Paran *et al.* (1991) proposed that RAPDs may be mapped to areas of the genome containing repetitive DNA which could not previously be examined using RFLPs.

RAPDs are scored directly from agarose gels. The lower resolution of such gels may produce a higher probability of error in the scoring of bands versus probing in RFLPs. Weeden *et al.* (1992) observed an error rate of 4% in the scoring of phenotypes in their examination of 169 F_2 individuals of pea using eight RAPD primers. Errors were attributed to poor amplification and difficulty in the scoring of bands (Weeden *et al.*, 1992). Weeden proposed that the poor amplification of some samples may have been due to an inhibitor of elongation isolated with the DNA. This inhibition was more pronounced with larger amplification products (Weeden *et al.*, 1992). Such high error rates impede phylogenetic studies using RAPD data (Weeden *et al.*, 1992).

Linkage was identified by Weeden *et al.* (1992) for 22 out of 27 polymorphisms in pea with well-characterized morphological or molecular markers. A random generation of fragments is unlikely to produce segregation patterns with such strong linkage with unrelated genetic markers (Weeden *et al.*, 1992). As most of the RAPD markers reflected real genetic variation, Weeden *et al.* (1992) determined that RAPDs were useful for mapping studies but that scoring errors would lead to increased map distances between adjacent markers and the possibility of minor changes in marker order in small regions of the genome. Weeden *et al.* (1992) proposed that scoring errors could be corrected by performing duplicate analyses and inspecting for an excess of double recombinants in regions of the genome where markers are at sufficient density.

Tingey *et al.* (1992) examined advances in the utilization of RAPDs for genetic analysis, showing the utility of RAPDs for the production of genetic maps. Genetic maps for *Arabidopsis thaliana* (Reiter *et al.*, 1992) and loblolly pine (Chaparro *et al.*, 1992) have recently been constructed using RAPD markers.

Although RAPDs can be detected more quickly than RFLPs, the markers generated are usually dominant. Of 31 polymorphisms detected between 27 inbred barley lines, one polymorphism revealed two codominant amplification products after Southern analysis (Tinker *et al.*, 1993). The polymorphic band (RAPD marker) has a high probability of containing repetitive DNA. From nine RAPD markers in lettuce, eight contained repetitive DNA as determined by dot blot and Southern analysis (Paran and Michelmore, 1993).

To convert RAPD markers into genomic probes necessary for chromosome walking, pairs of sequence characterized amplified region (SCAR) primers of 24 bp were produced based on the sequence at each end of the RAPD amplification products (Paran and Michelmore, 1993). SCAR primers produce single amplification products by PCR, sometimes generating length polymorphisms, converting dominant RAPD markers into codominant SCARs (Paran and Michelmore, 1993). Of nine sets of specifically designed SCAR primers produced by Paran and Michelmore (1993), three displayed length polymorphisms useful as codominant genetic markers. Screening of a YAC library using PCR has been described (Green and Olson, 1990), allowing PCR-based markers to be used in chromosome walking. SCARs tightly linked to resistance genes should prove useful in genomic walking experiments.

At present, polymorphisms must be examined for linkage to target genes using segregating populations. Screening for isozymes, RFLPs, or RAPDs as molecular markers linked to loci of interest would be optimized if one could increase the probability that they were linked to such loci. Two methods of enriching for linkage between polymorphic loci and the target region in the mapping of plant genes are NILs and bulked segregant analysis.

2.2.2 Populations useful for detection of molecular markers

(i) Near-isogenic lines (NILs)

Isogenic lines are derived from gene mutation in a pure line (Stam and Zeven, 1981). Backcrossing is used to produce NILs. In recurrent backcrossing, parts of the donor genome unlinked to the locus of interest will be introgressed into the recipient genome (Young and Tanksley, 1989). With successive backcrosses, the amount of DNA introgressed from the donor but unlinked to the target locus is reduced by a factor of two. By the eighth backcross generation, less than 0.2% of the unlinked donor genome will remain in the progeny (Young and Tanksley, 1989).

Paran *et al.* (1991) used NILs to identify RFLP and RAPD markers linked to *Dm* genes in lettuce. Paran *et al.* (1991) determined that half of the markers polymorphic for a pair of NILs should be linked to the *Dm* region in *Lactuca sativa* if the NILs were the result of five backcrosses. To reduce the number of markers linked to introgressed regions other than the target locus, Paran *et al.* (1991) combined pairs of NILs.

Six to eight years of backcrossing are required to produce NILs in lettuce. The

time for NIL production depends on species generation time. In *Arabidopsis thaliana* NILs can be produced within two years. The number of backcrosses is determined by the minimum allowable amount of donor DNA unlinked to the target locus (Young and Tanksley, 1989). The backcrossing process is difficult in self-pollinating species such as *L. sativa*. In the lettuce flower, a tube of fused anthers deposits pollen on the inside surface as the style elongates (Ryder, 1983). Careful washing of the stigma is necessary to prevent self-pollination when producing crosses between lettuce cultivars.

(ii) Bulked segregant analysis

Bulked segregant analysis has recently been described by Michelmore *et al.* (1991), enabling the identification of markers linked to a target locus using a single segregating population and negating the need to produce NILs.

In bulked segregant analysis, bulks of DNA are created around a single locus using a segregating population originating from a single cross (Michelmore *et al.*, 1991). Each bulk is a pool of DNA from individuals of identical genotype for the target locus but which are random at all loci unlinked to the region of interest. Bulks are screened for genetic polymorphisms using RFLP or RAPD techniques.

Bulks can be created for any locus in a shorter time than NILs. For example, in the case of *L. sativa*, bulks were produced within two years starting from a cross between cultivars carrying the traits of interest. F_1 seeds from a cross of the appropriate individuals are grown and selfed. Each F_1 individual produces an F_2 population segregating for the target locus. F_2 plants are screened for the phenotype of interest.

Polymorphisms detected with the resultant bulks are in *cis* to the target region as heterozygotes for the target locus are included in the bulks (Michelmore *et al.*, 1991). For polymorphisms in *cis* and *trans* to the target locus, the F_2 generation is selfed and F_3 families phenotyped to obtain F_2 genotypes so that F_2 heterozygotes are excluded from the bulks (Michelmore *et al.*, 1991).

Detection of polymorphisms genetically linked to the target region depends on bulk size and on the method chosen for polymorphism detection (RFLP or RAPDs). Using the equation $2(1-[1/4]^n)(1/4)^n$, where n is the number of individuals per bulk, bulks containing 10 individuals have a probability of producing a dominant RAPD marker unlinked to the target locus of 2×10^{-6} (Michelmore *et al.*, 1991). In bulks of adequate size (i.e. 10 or more individuals per bulk), all markers detected will segregate with the target locus and can be mapped (Michelmore *et al.*, 1991).

3. Turnip mosaic virus

3.1 Characterization

TuMV is a member of the potyvirus family of plant viruses, as characterized by the type member, potato virus Y (Ward and Shukla, 1991). This group has at least 180 classified members and is the largest and most economically important group of plant viruses (Ward and Shukla, 1991). TuMV is long and filamentous, with a length of 700-800 nm and a diameter of 12-13 nm (Smith, 1972). Pinwheel inclusion bodies are formed in the cytoplasm of TuMV-infected plants (McDonald and Hiebert, 1975). Up to 2000 units of a single coat protein subunit are arranged at a helical pitch of 3.4 nm

along the length of the potyviral RNA, producing the distinctive long, flexuous capsomere enclosing the genome (Hollings and Brunt, 1981).

Polyclonal and monoclonal antibodies have been produced to native TuMV proteins purified from infected plants (Raptis, 1975; Horsewood *et al.*, 1991). The sequencing and cloning of the TuMV genome facilitated the production of antibodies to TuMV using viral proteins expressed in *E. coli*. TuMV cDNA, necessary for cloning and sequencing, was produced by reverse-transcriptase PCR with degenerate primers designed from conserved areas of five previously sequenced potyviruses (Nicolas and Laliberté, 1991).

TuMV contains a single, positive strand RNA, consisting of 9830 nucleotides (Nicolas and Laliberté, 1992). Upon infection, a single potyviral polyprotein is produced using viral RNA as an mRNA template. This polyprotein is subsequently cleaved to produce various proteins necessary for replication (Allison *et al.*, 1985).

The genome of TuMV has recently been fully sequenced and various cistrons such as the NIa and coat protein have been cloned into expression vectors for protein expression and subsequent antibody production (Tremblay *et al.*, 1990; Nicolas and Laliberté, 1992; Laliberté *et al.*, 1992; Robbins *et al.*, submitted).

3.4 Host range

TuMV is the most important viral pathogen of cruciferous crops (Shattuck *et al.*, 1989). Among those species susceptible to TuMV are *Brassica perviridis*, *B. pekinensis*, and *B. napus*, all of which display systemic mosaic and stunting upon infection

(Tomlinson, 1970; Tomlinson and Ward, 1978). TuMV is transmitted by aphids in a non-persistent manner (Hollings and Brunt, 1981). Several aphid species can transmit TuMV, making it difficult to control its spread using aphicides (Tomlinson, 1970).

No complete immunity to TuMV has been found in the *Brassicaceae*. Complete immunity is defined in this case as resistance heritable at a single genetic locus which fails to display symptoms of infection or a HR or presence of virus by ELISA. Walkey and Neely (1980) found no cultivars immune to TuMV in an examination of white cabbage (*Brassica oleracea*). Cultivars were observed which were highly resistant in the field, with little or no symptoms and no development of internal necrosis during cold storage. Pink and Walkey (1990) examined some of these resistant cultivars for heritability of resistance to TuMV. Resistance in white cabbage to external necrosis caused by TuMV had a genetic component as shown by heritability in different cultivars ranging from 41% to 48%. Resistance to internal necrosis was not genetically determined (Pink and Walkey, 1990).

Tomlinson and Ward (1982) described two lines of *Brassica napus*, No.165 and No.181, which remained symptomless after self-pollination and inoculation of a British strain of TuMV of the first inbred generation (I_1). Seventy I_1 progeny inoculated twice with TuMV produced no symptoms (Tomlinson and Ward, 1982). Transmission electron microscopy of these plants failed to show the presence of TuMV particles and no local lesions were produced after inoculation of *Chenopodium quinoa* with extracts from these plants (Tomlinson and Ward, 1982). The second generation progeny of the I_1 plants were symptomless (Tomlinson and Ward, 1982). Shattuck and Stobbs (1987) later

determined that line 165 was not immune to the Ontario strain of TuMV, with mild chlorotic foliage symptoms and ELISA revealing the presence of TuMV. Shattuck and Stobbs (1987) described cv. Sensation to be immune to the Ontario isolate of TuMV. Further examinations involving other TuMV isolates and segregation studies are required.

Aside from cruciferous crops, TuMV infects a wide range of plant species including members of the families: *Aizoaceae*, *Amaranthaceae*, *Boraginaceae*, *Campanulaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Compositae*, *Euphorbiaceae*, *Geraniceae*, *Leguminosae*, *Papaveraceae*, *Portulaceae*, *Scrophulariaceae*, *Solanaceae*, and *Umbelliferae* (Zink and Duffus, 1969; Horváth, 1979). Symptoms produced on TuMV-susceptible species include: *Chenopodium quinoa* (local lesions and systemic mosaic) (Smith, 1972), *Chenopodium amaranticolor* (brown-rimmed lesions) (Smith, 1972), and *Nicotiana glutinosa* (systemic mosaic, stunting, occasionally lethal) (Smith, 1972).

TuMV infection of *Lactuca sativa* cv. Calmar produced systemic mosaic, severe stunting, circular chlorotic areas, and was occasionally lethal (Zink and Duffus, 1969). Duffus and Zink (1969) observed that it was the circular lesions and lack of yellowing or tip-roll which distingished TuMV from another potyvirus-induced disease of lettuce caused by LMV. Zink and Duffus (1969) noted that, of 79 cultivars of *L. sativa* examined for resistance to TuMV and mildew, cultivars were either susceptible or completely resistant to TuMV, with no segregation observed within a population of a given cultivar. TuMV-susceptible cultivars were mildew-resistant crisphead types: cv. Calmar, E-4, Imperial 410, Imperial Triumph, Valrio, Valtemp, and Valverde (Zink and

Duffus, 1969).

Resistance to TuMV cannot be introduced into the *Brassicaceae* from lettuce by traditional breeding programs due to incompatibility. A gene conferring resistance in one species may be functional in another if barriers to interspecific crosses were circumvented (Keen, 1990). Keen (1990) suggests that, because avirulence genes from one pathovar can function in different pathovars, the same resistance gene may function in different plant species. Map-based cloning of such resistance and *Agrobacterium*-mediated transformation offer a means of transferring traits such as resistance between incompatible species.

4. *Lactuca sativa*-turnip mosaic virus interactions

4.1 The resistance gene, *Tu*

Tu is a single dominant gene conferring resistance to TuMV on *L. sativa* (Zink and Duffus, 1970). From an examination of the pedigrees of the TuMV-susceptible cultivars of *L. sativa*, it was determined that susceptibility to TuMV (i.e. the *tu* allele) was introduced into *L. sativa* from P.I. 91532 and P.I. 177418 (Zink and Duffus, 1969). It is not known how *Tu* confers resistance to TuMV but no HR has been reported. All TuMV-resistant cultivars of *L. sativa* reported by Zink and Duffus (1969) were completely resistant, with no local lesion development and no recovery of virus after TuMV inoculation. A dominant gene which does not produce a HR upon infection does not fit Fraser's (1987) positive model of resistance. No virulent isolates of TuMV which overcome *Tu* have yet been reported, hence the possibility of a gene-for-gene interaction

has not been examined.

4.2 Linkage of *Tu* and *Dm5/8*

Zink and Duffus (1969) proposed that the allele for mildew resistance, *Dm*, and the allele for TuMV susceptibility, *tu*, were linked in trans as none of the *L. sativa* cultivars were susceptible to both TuMV and downy mildew. In a subsequent examination, Zink and Duffus (1970) determined, using data from eight F_2 progenies of crosses between TuMV-susceptible, mildew-resistant, and TuMV-resistant, mildew susceptible parents that *tu* was linked to *Dm*. The F_1 progeny from these crosses were TuMV and mildew resistant (Zink and Duffus, 1970). Of 3,682 F_2 plants assayed, 2,773 were TuMV-resistant and 909 were TuMV-susceptible (Zink and Duffus, 1970). A total of 2,788 mildew-resistant and 904 mildew-susceptible plants were observed in the F_2 (Zink and Duffus, 1970). It was concluded that resistance to TuMV and downy mildew were each controlled by a single dominant gene, designated as *Tu* and *Dm* (Zink and Duffus, 1970). The distance between these two loci was estimated at 12.5 map units (Zink and Duffus, 1970).

Landry *et al.*(1987) created the genetic map of lettuce using RFLPs. The orientation of *Tu* relative to *Dm5/8* on the map of *L. sativa* was not known. The area around *Dm5/8* on the genetic map of L. sativa has many RFLP and RAPD markers (Kesseli *et al.*, unpublished; Kesseli *et al.*, 1991). As *Tu* is close to *Dm5/8*, it is possible that one or more of these markers is tightly linked to *Tu*. Assigning *Tu* a map location is the first step towards the eventual cloning and characterization of the

resistance mechanism involved. New molecular markers found using bulked segregant analysis may be required if *Tu* falls in a genomic area without tightly linked flanking markers.

III. Materials and Methods

1. Identification of TuMV infection in plants

1.1 Optimization of inoculation conditions for turnip mosaic virus

Different dilutions of crude extract from TuMV-infected *Brassica perviridis* were produced using 0.1 M KPO_4 buffer, pH 7.5. The concentrations of infected leaf examined for inoculations, using the above buffer, were: 0.50 g/ml, 0.25 g/ml, and 0.11 g/ml. Each dilution was examined for infectivity by indirect enzyme-linked immunosorbent assay (ELISA), described below, on a population of 12 TuMV-susceptible *Lactuca sativa*, cv. Calmar. Five mock-inoculated Calmar individuals served as controls. The dilution which produced the minimum number of non-infected individuals was chosen as optimal for further experimentation. Ten Calmar individuals were then inoculated either once at the three-leaf stage, once at the 5-leaf stage, at both the 3 and 5-leaf stages, or at both the 5 and 7-leaf stages with a 0.25 g/ml suspension of TuMV-infected *B. perviridis* diluted in 0.1 M KPO_4 pH 7.5. TuMV-resistant *L. sativa*, cv. Kordaat, was inoculated on the same schedule as Calmar. A population of 13 mock-inoculated Calmar were used as controls. Three weeks after the final inoculation all plants were tested using indirect ELISA as described.

1.2 Turnip mosaic virus propagation

The TuMV isolate described by Tremblay *et al.* (1990) was propagated on *Brassica perviridis* under greenhouse conditions. Crude extracts were prepared by

grinding infected leaf material (0.25 g/ml) in 0.1 M KPO_4 pH 7.5, and used for inoculations. Inoculations were performed by dusting the leaf surface with carborundum and rubbing with a cotton swab saturated with crude extract from infected leaf tissue. Nine indicator plants (*Chenopodium quinoa*, *C. amaranticolor*, *B. perviridis*, *B. napus*, *B. pekinensis*, *Nicotiana glutinosa*, and *L. sativa* cultivars Calmar, Cobbham Green, and Kordaat) were used to verify the identity and the purity of the TuMV isolate used.

1.3 Virus purification

TuMV was purified from *B. perviridis* according to Choi *et al.* (1977). One-hundred grams of frozen TuMV-infected leaves were homogenized with 100 ml 0.5 M KPO_4 buffer (pH 7.5) containing 0.01 M Na_2EDTA and 0.1% mercaptoethanol and filtered through Miracloth (Calbiochem). Crude sap was centrifuged at 3 200 x g for 10 min. To the supernatant, 1% Triton X-100, 4% polyethylene glycol (PEG, m.w. 6000), and 0.1 M NaCl were added, the mixture was stirred at room temperature for 3 hours, and centrifuged at 8 500 x g for 10 min. The pellet was resuspended in 0.5 M KPO_4 (pH 7.5) containing 0.01 M $MgCl_2$ and centrifuged at 8 500 x g for 10 min. The supernatant was centrifuged at 65 900 x g for 1.5 hours, the pellet resuspended and centrifuged as in the previous step, and the resulting supernatant layered onto a 10-40% step sucrose density-gradient. The sucrose gradient was then spun for 2 hours at 61 000 x g. The blue-grey band approximately 2 cm from the top of the gradient, in the 10% sucrose layer, was diluted in 0.01 M KPO_4 (pH 7.0) and centrifuged at 69 600 x g for 1.5 hours. The resulting pellet was resuspended in 0.01 M KPO_4 (pH 7.0) and

centrifuged at 8 500 x g for 10 min. The supernatant containing purified virus was stored at -70°C.

1.4 Transmission electron microscopy

A 20 μl drop of purified virus (see above) from TuMV-infected *B. perviridis* was placed on Collodion-coated and carbon stabilized 200 mesh copper grids. Samples were allowed to partially dry for 2-5 min onto the grids. The remaining liquid was then removed using filter paper and the grid washed twice with dH_2O. Twenty microliters of 2% phosphotungstic acid, pH 7.0, was then added to the grid and removed after 2 min using filter paper followed by five washes using dH_2O. Grids were then placed in a Carl Zeiss Model EM9 transmission electron microscope for examination and photography.

1.5 Expression of the TuMV CP in *E. coli*.

A cDNA copy of the fully sequenced TuMV coat protein (CP) gene (Nicolas and Laliberté, 1990) was cloned into the pET-11-d expression vector by J.-F. Laliberté (Institut Armand Frappier, Laval, Québec). *E. coli* strain BL21 (DE3) harbouring the pET-CP plasmid, to create strain MR1, was obtained from J.-F. Laliberté and grown in Luria Broth (LB) with 100 μg/ml ampicillin (LB+amp) at 37°C. Fifty microliters were taken from saturated cultures grown in 3 ml of LB+amp at 37°C overnight and added to 50 ml LB+amp in 250 ml sterile flasks and incubated shaking until the OD_{600} reached 0.5. At OD_{600} of 0.5, 0.4 mM IPTG (isopropylthio-ß-galactoside) was added to promote transcription of the TuMV-CP gene and the cells were incubated for an additional two

hours after which they were harvested by centrifugation at 7 000 x g for 10 min.

1.6 Isolation of inclusion bodies containing recombinant TuMV-CP

TuMV CP was isolated from the MR1 strain of *E. coli* by cell disruption with lysozyme and differential centrifugation of the TuMV-CP inclusion bodies according to Harlow and Lane (1988). Bacterial pellets were resuspended in resuspension buffer (0.1 M NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0) to 10 % of the original volume (i.e. to 5 ml). Lysozyme was then added to a final concentration of 2 mg/ml. After 20 min of incubation at room temperature, the tubes were centrifuged at 5 000 x g for 10 min and the supernatant was discarded. Pellets were resuspended in ice cold resuspension buffer containing 0.1 % sodium deoxycholate and incubated on ice for 10 min. $MgCl_2$ was added to a final concentration of 8 mM and DNaseI to a final concentration of 10 μg/ml. This mixture was incubated at 4°C with occasional mixing until viscosity was reduced. Inclusion bodies were recovered by centrifugation at 10 000 g for 10 min. Pellets were washed twice in resuspension buffer containing 1 % NP-40 and centrifuged at 10 000 x g for 10 min. Pellets were then resuspended in 0.4 ml of sterile TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and stored at -20°C.

1.7 Polyacrylamide gel electrophoresis purification of TuMV-CP and antibody production

Proteins found in the inclusion bodies were left in TE buffer and electrophoretically separated on a 12.5% polyacrylamide gel (PAG) under denaturing

conditions (1 M urea and 0.4% SDS [sodium dodecyl sulfate]; Adams, 1989). Loading buffer (63 mM Tris-Cl pH 6.8, 10% glycerol, 2% SDS, 1 M urea and 0.05% bromophenol blue) was added in a 1:5 dilution to protein samples which were then denatured for 5 min at 100°C. Samples were loaded onto SDS-PAG composed of a stacking gel (0.125 M Tris.Cl, pH 6.8; 0.1% SDS [w/v]; 4.2% acrylamide/bis [w/v]; 0.05% fresh ammonium persulfate; 0.1% TEMED [N,N,N',N'-tetramethylethylenediamine]) and a separating gel (0.375 M Tris.Cl, pH 8.8; 0.1% SDS, 12.5% acrylamide/bis [w/v]; 0.05% fresh ammonium persulfate; 0.05% TEMED). Low molecular weight protein markers (Pharmacia) were used to determine molecular weights of proteins on the gel. The markers were: Phosphorylase b, 94 000 kDa; Bovine Serum Albumin (BSA), 67 000 kDa; Ovalbumin, 43 000 kDa; Carbonic Anhydrase, 30 000 kDa, Soybean Trypsin Inhibitor, 20 100 kDa; and α-Lactalbumin, 14 400 kDa. Side strips from the gel were stained for 45 min in 40% methanol, 7% acetic acid and 0.1% Coomassie Blue R250, and destained for 15 min in 40% methanol and 7% acetic acid. The band at approximately 34 kDa was cut from the unstained gel, pulverized by several passages in a 26G needle, emulsified in Freund's incomplete adjuvant (Difco) and injected intramuscularly into 8 week old female rabbits according to Vaitukaitis (1981). Three injections were performed at ten day intervals. Ten days after the third injection, serum was collected and stored at -70°C.

1.8 Immunodetection

An indirect ELISA assay based on the protocol of Clarke *et al.* (1986) was used

for the detection of TuMV CP. All incubations were at room temperature. Wells were washed six times between each step using phosphate buffered saline (PBS; 0.137 M NaCl, 27 mM KCl, 43 mM Na_2PO_4, 14 mM KH_2PO_4, pH 7.0) containing 0.05% Tween-20 (PBST). Leaf samples were ground in extraction buffer (0.150 M Na_2CO_3, 35 mM $NaHCO_3$ and 2% polyvinylpyrolidone (PVP) 44,000) at a ratio of 0.05 g of leaf per of millilitre buffer. Samples were centrifuged for one minute at 14 000 x g. Microtiter plate wells (Falcon) were blocked with PBST containing 2% bovine serum albumin (BSA). Anti-TuMV CP diluted 1:1000 in PBST buffer containing 0.2% BSA and 2% PVP was bound to the CP antigen. Antigen-antibody complexes were detected with goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) diluted 1:5000 in the same buffer. Substrate buffer (9.7% diethanolamine, pH 9.8) containing 1 mg/ml p-nitrophenyl phosphate (Sigma) was added and color intensity was measured at 405 nm.

1.9 Statistical analysis

A baseline for minimum positive values was produced using ELISA of the negative controls. Between 5 and 12 negative controls were included per cultivar per experiment. The upper limit for negative values was obtained using four standard deviations above the mean for the negative controls. Any plants with ELISA values above this baseline were scored as positive for TuMV-infection.

1.10 Immunoblotting

Infected and non-infected leaf extracts were prepared by grinding 0.25 g of leaf

in one ml of buffer (0.1 M Tris-Cl pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 0.1% mercaptoethanol). Protein was quantitated using a Coomassie Blue dye-binding assay (Bio-Rad). Samples were added to 40 μl of dye-binding reagent (Bio-Rad) and 160 μl H_2O in Falcon microtiter plates. Plate readings at OD_{595} were taken between 5 and 30 minutes after the addition of all reagents. A range of 5 different concentrations, 2 μg/μl, 4 μg/μl, 6 μg/μl, and 10 μg/μl of BSA were used to produce a standard curve of protein in this manner. After quantitation, the appropriate amounts of samples were diluted in loading buffer as previously described and run on a 15% polyacrylamide gel under denaturing conditions as described by Gallagher *et al.* (1989). All conditions for SDS-PAGE were as previously described except that 15% bis/acrylamide was used in the separating gel instead of 12.5%. Protein was transferred electrophoretically to nitrocellulose using a Bio-Rad protein transfer apparatus at 14 V for 12-16 hours. Anti-TuMV serum was diluted 1:800 in PBS containing 1% BSA. Goat anti-rabbit IgG linked to alkaline phosphatase (Bio-Rad) at a 1:3000 dilution in PBS and a nitroblue tetrazolium-based assay (Bio-Rad) were used for antigen-antibody complex detection (Gallagher *et al.*, 1989).

2. Identification of polymorphisms linked to *Tu*

2.1 Plant genomic DNA purification

DNA extractions were performed by the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987). Five grams of fresh leaf material were homogenized with 15 ml of CTAB- (1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-Cl, 0.2

% (v/v) 2-mercaptoethanol, pH 8.0) solution in a Waring blender. Five milliliters of 4X CTAB+ (8% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-Cl, 0.2% (v/v) 2-mercaptoethanol, pH 8.0) solution was then added and the homogenate incubated in a 60°C water bath for 30 min, swirling occasionally. Chloroform-isoamyl alcohol (24:1) was added in a 1:1 ratio to the homogenate and the mixture emulsified and centrifuged at 7 000 x g for 5 min. The aqueous phase was added to 2/3 volume of cold isopropanol and incubated for at least 30 min at -20°C to precipitate the DNA. DNA was pelleted by centrifugation at 7 000 g for 10 min. The supernatant was then removed and the pellet washed with wash buffer (10 mM ammonium acetate, 76% ethanol). DNA was vacuum dried and resuspended in sterile TE and stored at -20°C.

2.2 Agarose gel electrophoresis

Agarose gels of 1.2% were prepared by melting the appropriate amount of agarose in 0.75 X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were poured into a gel bed containing teflon combs for the formation of wells. To load samples, the comb was removed and 0.75 X TBE buffer added to the tank to cover the gel. To aid loading of DNA into wells, glycerol 10 X loading buffer (50% glycerol, 0.1 M NaEDTA, 1% (w/v) SDS, 0.2% bromophenol blue, 0.1% xylene cyanol) was added to DNA samples. After samples were loaded, a current of 2.9 V/cm was applied. Gels were stained with 0.5 μg/ml ethidium bromide and visualized under ultraviolet light.

2.3 Production of bulks for the *Tu* locus

Eighty F_2 plants originating from a cross between Calmar as the male parent (susceptible to TuMV) and Kordaat as the female parent (resistant) were planted and grown under greenhouse conditions in 4 cm pots containing Promix artificial soil. Plants were fertilized at monthly intervals. The fungicide Benlate was sprayed once at three weeks at a concentration of 1 mg/ml to prevent *Botrytis* infection. Leaf material was taken from each F_2 individual and frozen at -70°C. When the plants had bolted at approximately three months of age, and before flowering, all were covered with fine-mesh material to prevent cross-fertilization. Seed was harvested and 18 F_3 plants from each F_2 individual were planted in flats and treated as above. At the three-leaf and again at the 5-leaf stage, and using inoculum containing 0.25 g/ml of TuMV-infected *B. perviridis* in 0.1 M KPO_4, pH 7.5, all plants were inoculated (see section III.1.2). Three weeks after the final inoculation, all plants were tested for TuMV-infection by R. Singh using ELISA as described (section III.1.8).

2.4 Random amplified polymorphic DNA

Bulked samples of DNA, from plants characterized as homozygous resistant or susceptible to downy mildew, were created by Michelmore *et al.* (1991) using purified DNA of F_2 individuals from a cross between cultivars Calmar and Kordaat. These bulks were used for RAPD-PCR as described by Williams *et al.* (1991) using random decamers (R.J. Carlson, Univ. of British Columbia). Each amplification reaction contained approximately 20 ng of genomic DNA, 240 nM primer, 100 μM of each dNTP, 10 X

reaction buffer (0.1 M Tris-Cl pH 8.8, 0.5 M KCl, 15 mM $MgCl_2$, 1% Triton) and 1.25 units of *Taq* polymerase (BRL). Reactions were performed in a Hybaid thermal cycler placed at 4 °C and programmed for 45 cycles of 1 min at 35 °C, 2 min at 72 °C, 1 min at 92 °C, preceded by an initial denaturation step of 94 °C for 5 min. Amplified DNA patterns were analyzed by 1.2% agarose gel electrophoresis and ethidium bromide staining.

2.5 Southern blotting

After separation and visualisation, RAPD-PCR products were transferred to Hybond N+ positively charged nylon membrane (Amersham) by alkaline capillary blotting (Wahl *et al.*, 1979; Meinkoth and Wahl, 1984). A blotting paper (3MM) wick was placed over a tray of 0.4M NaOH. The gel was placed upside down on the wick and a sheet of Hybond N+ membrane of the same dimension was put on top of the gel, followed by 3MM paper and a 10 cm high stack of paper towel. After a transfer of 24 hours the membrane was rinsed in 2 X SSC (0.3 M NaCl, 30 mM Na-citrate, pH 7.0).

2.6 Probe preparation

For nonradioactive probes, 50-100 ng of DNA were labelled with digoxigenin (Boehringer Mannheim). DNA for labelling was obtained from reamplified polymorphic bands of primers UBC517, UBC563, UBC599, or UBC675 isolated from agarose gel using Geneclean (Bio 101). Following the manufacturer's instructions, DNA was denatured at 100°C for 10 min, added to hexanucleotides, dNTP, and Klenow enzyme,

and incubated 20 hours at 37°C. The reaction was stopped using 25 mM EDTA, pH 8.0 and the probe precipitated using 0.5 M LiCl and 3 volumes of prechilled ethanol for 2 hours at -20°C. The probe was washed using cold 70% ethanol, dried under vacuum and dissolved in 50 ul TE buffer.

The polymorphic band amplified using primer UBC346 was cut from the gel and approximately 50 ng were labelled using the random priming method (Feinberg and Vogelstein, 1983) with ^{32}P-dCTP and T7 DNA polymerase as per the manufacturer's instructions (Pharmacia). Specific activity of the probe was determined by a scintillation counter.

2.7 Prehybridization and hybridization

Filters were placed in hybridization bottles with 50 ml of hybridization solution (5 X SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% N-lauroylsarcosine, 0.02% SDS (w/v), 50% formamide) and incubated at 42°C for 12 hours. For hybridization, prehybridization solution was discarded and 5 ml of prehybridization solution containing freshly denatured probe was added and allowed to hybridize at 42°C for 24 hours. After hybridization, filters were washed twice for 5 min with 100 ml of 2 X SSC, 0.1% SDS (w/v) and twice for 15 min at 68°C with 0.1 X SSC, 0.1% SDS (w/v). For nonradioactive probes, detection was as described by the manufacturer (Boehringer Mannheim). The radioactive probe was detected by exposure to X-ray film for 30 min.

3. Mapping of *Tu* and RAPD markers

The program Mapmaker 3.0 (Lander *et al.*, 1987) was used to map *Tu* relative to *Dm5/8* and to map new RAPD markers linked to *Tu*. Two and three-point mapping were carried out using an algorithm written into the program.

IV. Results and Discussion

1. Optimization of inoculation conditions

The TuMV strain sequenced by Nicolas and Laliberté (1992) was obtained and used for inoculations. TuMV was propagated on the TuMV-susceptible species *B. perviridis*. *B. perviridis* plants were inoculated with TuMV at the two leaf stage. The third to sixth leaves displayed severe mosaic with less stunting than later emerging leaves. *B. perviridis* was chosen for virus propagation instead of a TuMV-susceptible cultivar of *L. sativa* to minimize the probability of contamination with other viruses of lettuce, such as LMV, in the inoculum for the phenotyping experiments (see section IV.3). Seeds of *B. perviridis*, Tendergreen mustard spinach, were obtained from Northrup King Co. (Minneapolis, USA). The leaves of *B. perviridis* (third to sixth) yielded approximately 15 g/plant of TuMV-infected material. For virus purification, TuMV-infected leaves were harvested three weeks following TuMV-inoculation and stored at -70°C. Infected leaves were taken from each plant only once due to stunting caused by TuMV-infection. Five to twenty TuMV-infected *B. perviridis* were planted every two weeks and maintained under greenhouse conditions for use as inoculum. Frozen leaf material provided lower infectivity in inoculum as compared to freshly-infected *B. perviridis* leaves. The lower infectivity of frozen TuMV-infected leaves may have resulted from repeated freezing and thawing of the stock supply. Freshly infected leaf was used as inoculum in all experiments.

To orient *Tu* relative to *Dm*5/8, F_2 populations resulting from crosses between

TuMV-resistant and TuMV-susceptible cultivars of *L. sativa* were obtained from R.W. Michelmore. Twenty-three F_2 individuals from a cross between cv. Calmar (TuMV-susceptible) and cv. Cobbham Green (TuMV-resistant) were identified by R. W. Michelmore as recombinant near *Dm*5/8. Recombinant F_2 plants were the most informative for the location of *Tu*. Plants were defined as recombinant in the area of *Dm*5/8 if one or more crossover events occurred between homologous chromosomes of cv. Calmar and cv. Cobbham Green in the genetic region surrounding *Dm*5/8. The area of crossover can be narrowed by locating the transition point between alleles of one parent and allele types of the other parent. A data set for a recombinant individual could be A A A B B B, where each letter represents a genotype at a locus for a total of six loci (locus 1 through 6). In this individual, there has been a recombination (or crossover) event where the A genotype at locus 3 (like the cv. Calmar parent) changes to a B genotype at locus 4 (like the cv. Cobbham Green parent). A recombinant plant which is genetically cv. Calmar (*tutu*) on one side of *Dm*5/8 and cv. Cobbham Green (*TuTu*) on the other side of *Dm*5/8 (as determined using molecular or morphological markers in the area) and which is of genotype *TuTu* places *Tu* on the cv. Cobbham Green side of *Dm*5/8.

To locate *Tu* on the genetic map of *L. sativa*, the genotypes of F_2 recombinants at *Tu* were added to mapping information provided by R. W. Michelmore for markers in the area of *Dm*5/8 (table 1). F_3 seed from each F_2 recombinant were available. F_2 genotypes at *Tu* were found by phenotyping 10 to 18 F_3 individuals per F_2 recombinant. An ELISA test was used to determine F_3 phenotypes for TuMV. Errors in phenotyping

Table 1. Genotypes obtained from R. W. Michelmore in the region of the *Tu* locus for F_2 recombinant individuals from a cross between cvs. Calmar (TuMV susceptible) and Cobbham Green (TuMV resistant). Numbers in the left column represent F_2 individuals. Letters in the table represent genotypes A = homozygous Calmar, B = homozygous Cobbham Green, H = heterozygote, C = heterozygote or B, D = heterozygote or A.

F_2	*OPC08*	*plr*	*OPS12*	*OPX03*	*Dm5/8*	*OPD08*	*OPF12*	*OPM18*	*OPL08*	*OPY13*
8	C	B	B	C	B	B	B	B	B	D
11	C	B	B	C	B	B	D	D	D	D
12	C	B	B	C	B	B	B	B	B	B
15	A	H	H	C	H	D	D	D	D	D
27	C	H	A	A	A	D	D	D	D	D
28	C	A	D	A	A	D	D	D	D	D
34	C	H	D	C	H	D	D	D	D	B
35	C	-	D	C	H	D	D	D	D	B
37	C	H	D	C	H	D	D	D	D	D
38	C	H	D	H	B	B	B	B	B	B
40	C	H	D	C	H	D	D	B	B	B
62	C	H	D	H	B	B	B	B	B	B
85	C	H	D	A	A	D	D	D	D	D
87	A	A	D	A	H	D	D	D	D	D
95	C	H	-	A	H	D	D	D	D	D
106	C	H	D	C	H	D	D	D	D	B
108	C	H	A	A	A	D	D	D	D	D
132	A	H	H	C	H	D	D	D	D	D
134	C	H	D	C	H	D	D	B	B	B
140	C	H	D	C	H	D	D	B	B	B
144	C	H	D	C	H	D	B	B	B	B
145	C	H	D	C	H	B	B	B	B	B
149	C	H	D	C	A	D	D	D	D	D

F_3 families would affect F_2 genotyping and change the position of *Tu* on the genetic map of *L. sativa*. Conditions of inoculation were optimized to reduce errors in the phenotyping experiments.

Three different concentrations (0.50 g/ml, 0.25 g/ml, and 0.11 g/ml) of TuMV-infected *B. perviridis* in 0.1 M KPO_4 (pH 7.5) were examined for infectivity on cv. Calmar. A 0.25 g/ml concentration of leaf in KPO_4 buffer was recommended for inoculation using TuMV-infected *B. pekinensis* (Raptis, 1975). A higher (0.50 g/ml) and a lower (0.11 g/ml) concentration were examined in addition to 0.25 g/ml to determine the optimal amount of leaf in the inoculum. All concentrations of leaf in KPO_4 buffer were highly infective when manually inoculated onto Calmar (table 2). Horsewood *et al.* (1991) determined whether ELISA readings were positive or negative for TuMV-infection by establishing a threshold of negative values two standard deviations above the mean of the negative controls. Two standard deviations above the mean for five mock-inoculated Calmar controls was 0.054 ELISA units (OD_{405} after subtraction of background as determined using buffer controls within each plate) (table 2). All 10 individuals inoculated with 0.25 g/ml and 0.11 g/ml of TuMV-infected *B. perviridis* in KPO_4 buffer showed TuMV-infection as determined by ELISA using the negative threshold of 0.054 ELISA units (table 2). Visual symptoms of TuMV-infection, stunting, mosaic, and circular lesions on susceptible cultivars of *L. sativa* (figure 1b), correlated with ELISA values. The concentration of 0.25 g/ml of TuMV-infected *B. perviridis* was used for all further inoculations.

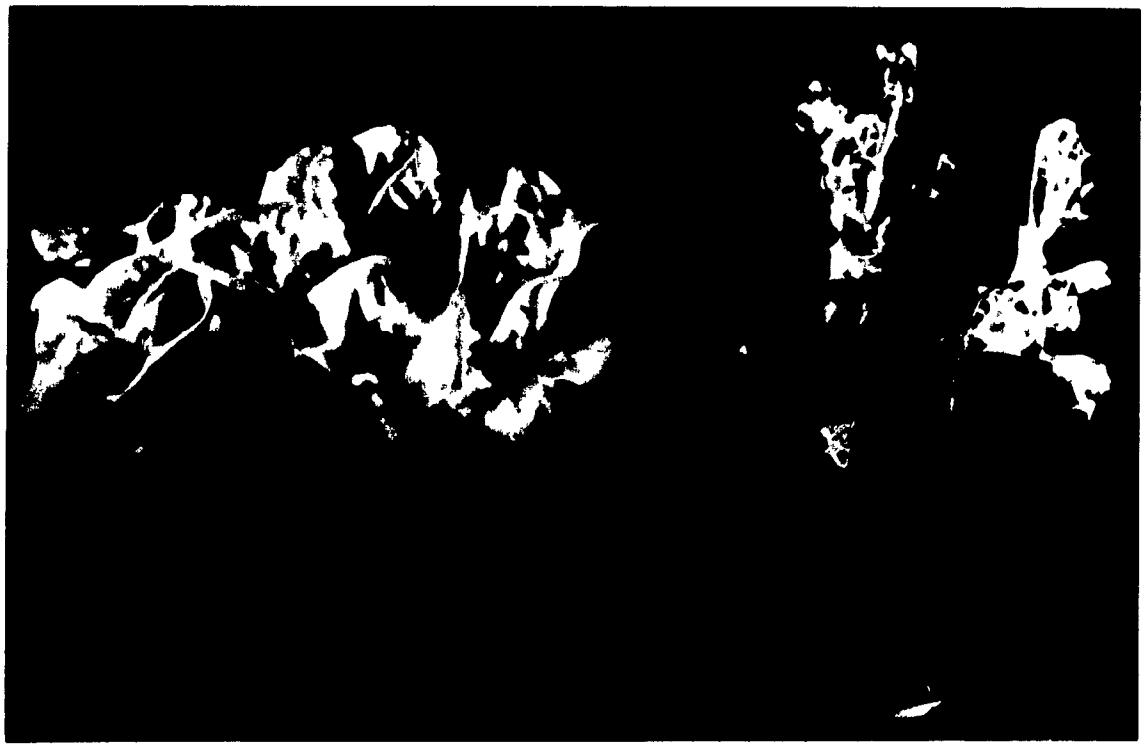
Ten individuals of cv. Calmar were inoculated at the 3-leaf, 5-leaf, 3 and 5-leaf,

Table 2. ELISA values for Calmar inoculated with 0.5 g/ml, 0.25 g/ml, or 0.115 g/ml of TuMV-infected *B. perviridis*. Numbers in the table represent ELISA values for each Calmar individual three weeks after inoculation. Mock are mock-inoculated Calmar controls.

0.5 g leaf/ml	0.25 g leaf/ml	0.11 g leaf/ml	mock
0.050*	0.108	0.100	0.034
0.493	0.444	0.308	0.041
0.143	0.090	0.486	0.039
0.104	0.365	0.276	0.052
0.086	0.096	0.070	0.038
0.103	0.529	0.397	$\bar{x}$=0.041
0.488	0.358	0.849	s=0.0068
0.364	0.081	0.683	$\bar{x}$+2s=0.054
0.750	0.324	0.510	$\bar{x}$+4s=0.068
0.964	0.393	0.231	
0.250	0.400	0.523	
0.400	0.304	0.395	

* ELISA values which were classified as negative for TuMV-infection using a maximum threshold for negative values four standard deviations above the mean of five mock-inoculated Calmar controls.

Figure 1. Symptoms on *L. sativa* cvs. Calmar and Kordaat after TuMV-inoculation. a. TuMV-resistant cv. of *L. sativa* (Kordaat) inoculated with TuMV at the three and five-leaf stage with 0.25 g/ml of TuMV-infected *B. perviridis*. b. TuMV-susceptible cv. Calmar inoculated as above.



A B

or 5 and 7-leaf stages to determine if the age of the plant and repetition of inoculation affected infection rates. Plants were scored as negative for TuMV-infection if the ELISA value was lower than the value two standard deviations above the mean for 12 mock-inoculated Calmar controls. Using 0.25 g/ml of TuMV-infected *B. perviridis* in buffer, it was found that earlier inoculations (3-leaf stage) repeated at the 5-leaf stage produced earlier and more severe symptoms with infectivity of 95% (19 out of 20 plants positive for TuMV-infection) for susceptible *L. sativa* cv. Calmar than a single inoculation at the 3-leaf stage producing infection rates of 90% (table 3a). Inoculations performed on older plants (5- and 7-leaf) produced symptoms later than on younger (3-leaf) plants. Although infection rates of cv. Calmar inoculated once at the 5-leaf stage were 95%, symptoms appeared later, as evidenced by the large number of ELISA values close to the threshold of 0.165 ELISA units (i.e. 0.189, 0.219, 0.221, 0.200 and 0.221) determined using the mock-inoculated controls (table 3a). One mock-inoculated Calmar control displayed an ELISA reading of 0.200 (table 3a). This value is above the threshold value of 0.165. Either this plant became infected with TuMV or there was high variability in the background levels of the negative controls. A negative threshold established using four standard deviations above the mean for the 12 mock-inoculated Calmar controls gives a value of 0.236. This value maintains infection rates of 95% using two inoculations (3- and 5-leaf) while lowering infection rates at the 5-leaf to 70% and at the 5- and 7-leaf stage to 80% (down from 90% using a threshold of two standard deviations above the mean). For early development of symptoms and accurate phenotyping, all subsequent inoculations were performed twice (at the 3-leaf and the 5-leaf stage) and ELISA values

Table 3a. ELISA values for Calmar inoculated with TuMV at the 3-, 5-, 3- and 5-, or 5- and 7-leaf stages. Replicates were performed for all treatments.

3 leaf rep 1	3 leaf rep 2	5 leaf rep 1	5 leaf rep 2	3 and 5 leaf,rep1	3 and 5 leaf,rep2	5 and 7 leaf,rep1	5 and 7 leaf,rep2	mock inoc.
0.329	0.090*	0.434	0.221*	0.932	0.581	0.669	0.300	0.076
0.958	0.080*	0.219*	0.200*	0.973	*****	0.493	0.219*	0.084
1.593	1.015	0.354	0.221*	0.753	0.950	0.496	0.474	0.087
0.914	0.889	0.567	0.487	1.065	0.949	0.271	0.457	0.084
1.569	0.703	0.189*	0.159*	0.836	*****	0.253	0.082*	0.074
1.170	0.901	0.357	0.236	1.310	0.567	0.268	0.228*	0.078
1.260	0.602	0.489	0.280	1.265	0.243	0.561	0.341	0.086
1.355	0.782	0.607	0.257	0.562	0.576	0.264	0.147*	0.200
1.542	0.858	0.612	0.310	0.119*	0.972	0.570	0.230	0.117
0.938	0.802	0.591	0.490	0.554	1.119	0.414	0.297	0.071
								0.075
								0.087

$\bar{x}$=0.093
s=0.036
$\bar{x}$+2s=0.165
$\bar{x}$+4s=0.236

**** ELISA value was greater than 2.000
* ELISA values classified as negative for TuMV-infection using a maximum threshold of negative values four standard deviations above the mean for 12 mock-inoculated controls.

evaluated using a threshold four standard deviations above the mean for mock-inoculated controls. All leaves of each individual were inoculated.

The resistant cv. Kordaat, inoculated with TuMV using crude extract of 0.25 g/ml *B. perviridis* at the 3- and 5- and 5- and 7-leaf stages, never displayed visible symptoms of TuMV infection and ELISA readings were similar for inoculated as for mock-inoculated Kordaat controls (table 3b, figure 1a). A negative threshold two standard deviations above the mean of the negative controls was not sufficient to prevent some Kordaat from being classified as positive for TuMV-infection (table 3b). A threshold of four standard deviations above the mean for six mock-inoculated Kordaat controls (0.124 ELISA units) ensured that all TuMV-inoculated Kordaat plants were scored as negative for TuMV-infection (table 3b). Further experiments comparing inoculation efficiencies of partially purified and purified TuMV with 0.25 g/ml of TuMV-*B. perviridis* in crude extract were deemed unnecessary as, with purification, infectivity of filamentous viruses such as TuMV tends to be lost due to aggregation of viral particles (Matthews, 1991).

Infection rates of 95% using 0.25 g/ml TuMV-infected *B. perviridis* resulted from a frequency of one error per 20 plants (table 3a). This error designates, by ELISA, a TuMV-susceptible plant as resistant to TuMV due to inefficiency of inoculation procedures. *Tu* is a dominant gene. F_2 heterozygotes, when selfed, produce, according to Mendelian segregation of phenotypes, a three:one ratio of *Tu_*:*tutu* F_3 individuals. A binomial equation at $p=0.75$ can be used to determine the probabilities of different F_3 phenotypic ratios, where p is the probability of a *Tutu* plant, when selfed, producing a

Table 3b. ELISA values for cv. Kordaat inoculated with TuMV at the 3- and 5- or 5- and 7-leaf stages. Replicates were done for both treatments.

3 and 5 leaf rep1	3 and 5 leaf rep2	5 and 7 leaf rep1	5 and 7 leaf rep2	mock inoculated
0.089	0.102	0.100	0.076	0.062
0.079	0.089	0.067	0.086	0.064
0.080	0.076	0.071	0.094	0.064
0.083	0.071	0.071	0.072	0.091
0.063	0.080	0.058	0.111	0.073
0.057	0.071	0.074	0.083	0.087
0.061	0.082	0.086	0.090	
0.088	0.083	0.078	0.078	$\bar{x}$=0.074
0.074	0.081	0.085	0.077	s=0.013
0.074	0.080	0.051	0.081	$\bar{x}$+4s=0.124

* All ELISA values in this table were classified as negative for TuMV-infection using a maximum threshold of negative values, four standard deviations above the mean of 6 Kordaat controls.

TuTu offspring. With x equal to the number of resistant offspring in a F_3 population of 10 plants, there is a high probability (P) of having eight resistant and two susceptible ($P_{(x=8)}$ of 0.282) or seven resistant and three susceptible ($P_{(x=7)}$ of 0.250) plants. The probability of one resistant and nine susceptible F_3 individuals resulting from a F_2 heterozygote is 2.86×10^{-5}. One susceptible and nine resistant F_3 plants have a binomial probability of 0.188. In the phenotyping experiments, one out of 20 plants will be falsely identified as TuMV-resistant (false negative) using a threshold four standard deviations above the mean of the negative controls. The probability is higher that a false negative (2.86×10^{-5}) will be recognized than a false positive (0.188) (i.e. a false negative will be re-examined because of its low probability). A negative threshold which is too low (such as two standard deviations above the mean of mock-inoculated controls), increases the chance of a *TuTu* F_2 recombinant being falsely scored as *Tutu* due to the presence of a false positive in the F_3 phenotyping population. F_3 recombinant families subsequently tested by ELISA were scored as either positive or negative for TuMV-infection based on whether the ELISA value was above (positive) or below (negative) the negative threshold.

For the phenotyping of F_3 families from F_2 individuals of the Calmar x Cobbham Green cross, the optimal conditions of inoculation were determined as 0.25 g/ml of TuMV-infected *B. perviridis* in 0.1 M KPO_4, pH 7.5 with plants inoculated on all leaves at the 3-leaf stage followed by a second identical inoculation at the 5-leaf stage. Optimization of conditions were performed using only Calmar and Cobbham Green. The same inoculation conditions were used in phenotyping F_3 recombinant families originating

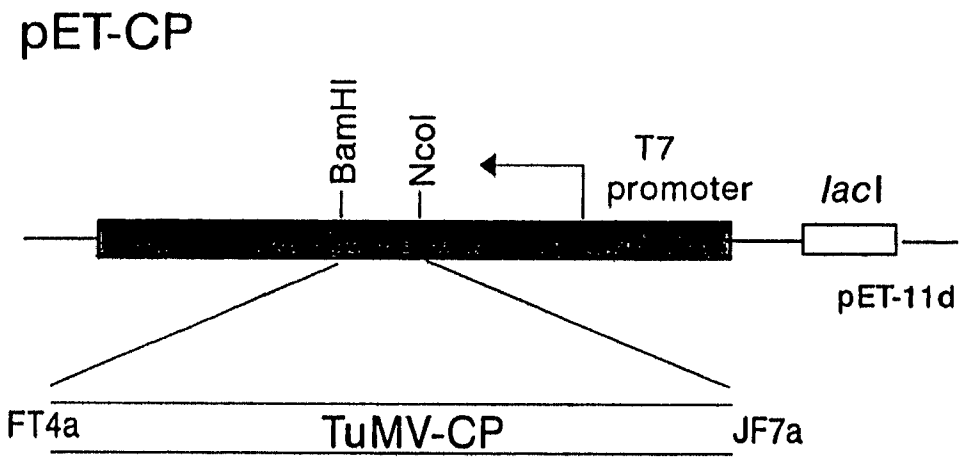
from a cross between Calmar and Kordaat (TuMV-resistant) and for bulk production around the *Tu* locus using F_3 families from F_2 individuals of a Calmar x Kordaat cross.

2. Expression of the TuMV CP in *E. coli* and antibody production

No commercial antibody was available for TuMV. Antibodies to TuMV had been produced (Raptis, 1975; Horsewood, 1991). These antibodies were made using CP purified from TuMV-infected plants. Polyclonal antibodies from plant-purified virus often contain cross-reacting antibodies to plant proteins, producing high background. ELISA using monoclonal antibodies may or may not be sensitive depending on the epitope to which the antibody is produced. A monoclonal antibody produced to one strain of a virus may not be as efficient when used for a different strain, depending on the similarities between strains at that epitope.

A reliable method for phenotyping F_3 recombinant families was needed. After optimizing inoculation conditions (section IV.1), the ELISA procedure was examined for sensitivity of TuMV-detection in virus-infected *L. sativa*. The genome of TuMV had recently been sequenced and various cistrons cloned into expression vectors such as pET-11-d (Nicolas and Laliberté, 1992; Studier et al., 1990). The CP cistron of this TuMV isolate had been cloned into pET-11-d by J.-F. Laliberté, forming pET-CP for high expression in *E. coli* (figure 2). CP protein expressed in *E. coli* has no contaminating plant proteins. Polyclonal antibodies, although not specific to a particular strain (the polyclonal antibody produced below reacts mildly to LMV CP) are faster to produce than monoclonal antibodies. Many monoclonal antibodies must be screened to find one which

Figure 2. pET-CP used for overexpression of cloned TuMV-CP. *E. coli* strain BL-21 was transformed with pET-CP. In BL-21 the T7 RNA polymerase gene is under the control of the *lacI*^{q} transcriptional regulator. Expression of TuMV-CP occurs with addition of IPTG.



FT4a 5'-TATAGTCTACCAggatccACTTCATAACCCCTGAAGGCC

JF7a 5'-GGTGTTGAGGCTTggatccGAAccatggCAGGTGAAACG

is sensitive and not cross-reacting to plant proteins (Horsewood *et al.*, 1991).

pET-CP was designed to express a protein with a theoretical molecular weight (MW) of 34 KDa comprising amino acid residues 2,876 to 3,863 of the TuMV polyprotein (Nicolas and Laliberté, 1992); all the codons between the ATG and the TAA belonged to the TuMV CP. pET-CP was introduced into strain BL21 of *E. coli* by J.-F. Laliberté where expression of the CP was regulated by the presence of IPTG: addition of IPTG to the medium induces transcription of the T7 RNA polymerase gene under the control of the $lacI^q$ promoter, the latter will then transcribe the CP gene located downstream of a T7 RNA polymerase promoter (Studier *et al.*, 1990). Upon induction with IPTG, almost all of the protein found in inclusion bodies consisted of TuMV CP, as determined by SDS-PAGE (figure 3). Protein preparations of cells induced with IPTG and harbouring pET-CP displayed a major protein at 34 kDa which was not present in cells containing the pET-11d vector without insert. The expressed TuMV-CP comigrated with the CP from a viral preparation of TuMV on SDS-PAGE (see below). The observed molecular weight of the *E. coli* produced protein (as determined by SDS-PAGE) was slightly larger than that predicted from the nucleotide sequence (Tremblay *et al.*, 1990). Anomalous behaviour of the CP of purified potyviruses in SDS-PAGE has been reported (Abo El-Nil *et al.*, 1977; Choi and Wakimoto, 1979; and Hiebert and McDonald, 1976).

The TuMV CP band was cut out of the gel and injected into rabbits to obtain a polyclonal antiserum. The antibodies detected one band on Western blots of SDS-PAGE separated proteins of untransformed BL-21 strain (figure 4, lane 1) or of transformed but

Figure 3. SDS-PAGE of TuMV-CP expressed and isolated from *E. coli*. Production of TuMV-CP was induced in the MR1 strain by addition of IPTG and inclusion bodies purified from cell lysate by differential centrifiugation. The major protein at approximately 34 KDa (see arrow) consists of TuMV-CP.

uninduced bacteria (figure 4, lane 2), suggesting that an *E. coli* protein of slightly lower molecular weight than the TuMV CP was recognized. *E. coli* proteins were co-purified in the inclusion bodies containing the TuMV-CP. One *E. coli* protein migrated closely with the TuMV-CP. The TuMV-CP band and the comigrating *E. coli* protein were cut from the gel after estimation of position using stained side-strips. This contaminating protein does not affect the sensitivity or specificity of the ELISA as no *E. coli* proteins are present in *L. sativa* (figure 4, lane 5).

An additional band of 38 kDa was detected in cells harbouring pET-CP induced by IPTG (figure 4, lane 3). The 38 kDa band corresponds to TuMV CP since a band of identical molecular weight is detected in TuMV-infected *B. perviridis* crude extracts (figure 4, lane 5), which is absent from healthy plants (figure 4, lane 4). Transmission electron microscopy indicated that a potyvirus-like particle was purified from TuMV-inoculated plants (figure 5). When the same viral preparation used for this Western blot was inoculated on diagnostic species (*L. sativa* cvs. Calmar, Kordaat and Cobbham Green, *Nicotiana glutinosa*, *Chenopodium quinoa* and *C. amaranticolor*, *B. perviridis*, *B. napus* and *B. pekinensis*) symptoms consistent with TuMV infections were produced. The same viral preparation was used to generate pET-CP. This confirmed that the serum did in fact recognize the CP from TuMV.

The antiserum was used to determine whether inoculated plants from F_3 individuals of crosses between cv. Calmar (TuMV-susceptible) and cv. Cobbham Green (TuMV-resistant) and cv. Calmar and cv. Kordaat (TuMV-resistant) were resistant or susceptible to TuMV. Resistance or susceptibility of F_3 families were used to determine

Figure 4. Immunodetection of TuMV-CP using antiserum raised against TuMV-CP expressed in *E. coli*. Protein extracts from strain BL-21 untransformed (lane 1), transformed with pET-CP but uninduced with IPTG (lane 2), transformed with pET-CP and induced to express TuMV-CP by addition of IPTG (lane 3) were separated by SDS-PAGE and transferred to nitrocellulose. Crude plant extracts from healthy (lane 4) and TuMV-infected plant material (lane 5) were also run in the same gel. In each lane, 0.5 μg of protein was loaded. The membrane was incubated with anti-TuMV serum followed by anti-IgG linked to alkaline phosphatase. The arrow indicates the band of TuMV-CP at 38 kDa.

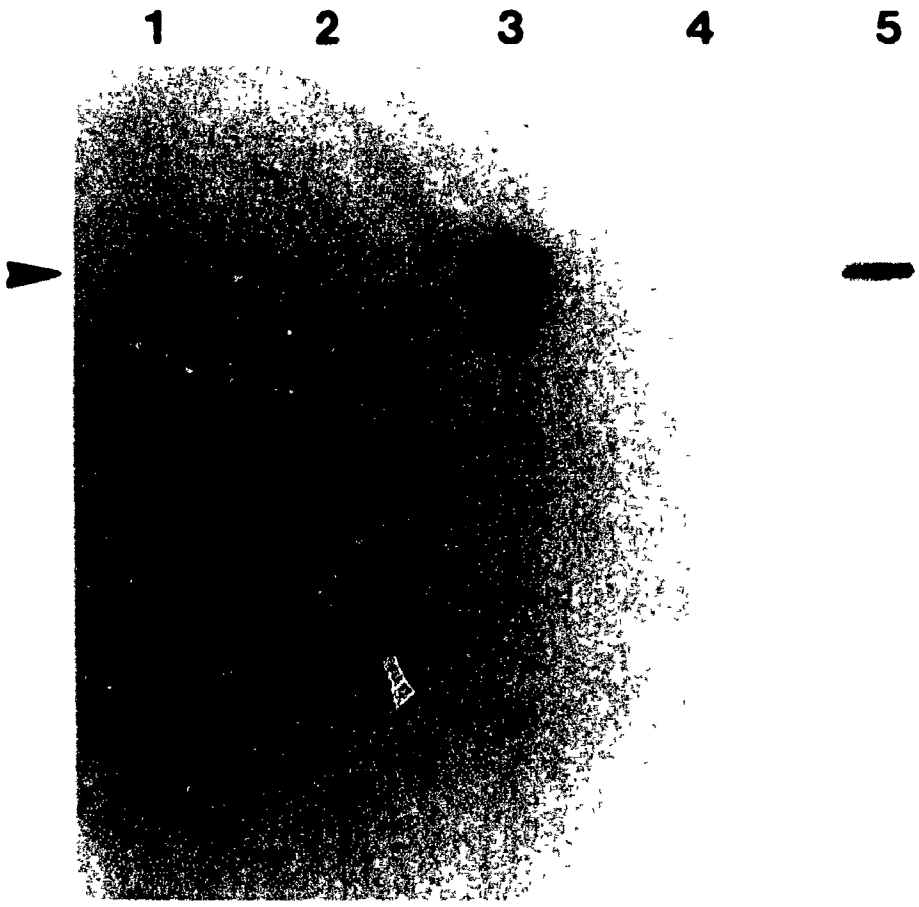

1
2
3
4
5

Figure 5. Transmission electron micrograph of TuMV-infected *B. perviridis*. TuMV was purified from *B. perviridis* and 20 μl partially dried onto the grid. After washing with H_2O, virus particles were stained with 0.2 M phosphotungstic acid, pH 6.2. Total magnification is 79,875 X actual size. Scale-bar (2 cm) equals 0.26 μm.

genotypes of F_2 progenitors from the above mapping populations at *Tu*.

3. Location of *Tu* on the *L. sativa* genome

The location of *Tu* in relation to markers identified by bulked segregant analysis and other previously mapped markers (Kesseli *et al.*, 1991; unpublished) was determined by establishing the genotype at the *Tu* locus for each F_2 individual identified as a recombinant in the genomic region surrounding *Dm*5/8. Zink and Duffus (1970) reported linkage at approximately 13 map units in *trans* between the *Dm*5/8 and the *Tu* loci in a cross involving *L. sativa* cvs. Calmar and Kordaat. Twenty-three F_3 families of a cross between cvs. Calmar (*tutu*) and Cobbham Green (*TuTu*) from F_2 plants that had been previously identified as recombinant in a region of approximately 20 cM were analyzed.

The mapping population consisted of a total of 142 F_2 individuals with 34 homozygous susceptible to TuMV, 27 homozygous resistant to TuMV, 55 heterozygotes, and 26 recombinant individuals in the area of *Dm5/8*. Only recombinant families in the *Dm5/8* region were phenotyped since they were the only informative individuals for mapping purposes.

Ten F_3 individuals from each of 23 F_2 families were inoculated with TuMV using 0.25 g/ml of TuMV-infected *B. perviridis* in 0.1 M KPO_4 buffer at both the 3- and 5-leaf stages. An ELISA protocol was used to determine whether the inoculated plants were resistant or susceptible to TuMV, in addition to the scoring of visual symptoms (data not shown). Analysis for segregation ratios of resistance to susceptibility within F_3 families allowed the genotype of progenitor F_2 plants to be determined (table 4a, 4b, and 4c).

If all F_3 plants were TuMV-susceptible, the F_2 parent was scored as A (Calmar=*tutu*) at the *Tu* locus. F_3 plants which were all resistant indicated a F_2 genotype at *Tu* of B (Kordaat=*TuTu*). The presence of both susceptible and resistant F_3 individuals indicated that the F_2 plant was H (Heterozygote=*Tutu*) (tables 4a, 4b, 4c).

All plants that displayed symptoms were found to contain virus by ELISA. Plants were scored as susceptible if they had an ELISA value above the negative threshold in one ELISA (first or second) and the third ELISA or in one ELISA and were subsequently scored as dead from viral infection in following ELISAs. Symptoms took longer to appear in some families (#85) and so three ELISAs, at an interval of three weeks between each ELISA, were necessary. Before the third ELISA analysis, plants were moved from the growth chamber to the greenhouse due to their large size. Insect pests and pathogens such as *Botrytis* as well as some plants being too close to the heating elements affected phenotypic ratios. For example, families #134, #144, and #145 showed consistently negative ELISA readings in the first two ELISAs for all F_3 individuals (table 4a, 4b) but, after transfer to the greenhouse, some plants died which were close to the heating element. A large period of time elapsed between the second and third ELISAs (plants were bolting by the third ELISA reading), increasing the chances of plants dying from causes other than TuMV, especially in the greenhouse. In approximately 20% of infected plants, visual symptoms could not be observed or were too faint to allow an unequivocal determination of whether the plant was resistant or susceptible.

The death of some F_3 plants in the greenhouse introduced potential false positives

Table 4a. ELISA values for recombinant F_3 families of a cross between Calmar and Cobbham Green Ten individuals of each of 23 families were inoculated with TuMV and assayed for TuMV-infection by ELISA 3 weeks post-inoculation. Blanks indicate missing data.

F_2 Parent	1	2	3	4	5	6	7	8	9	10
8	0.080*	0.139*	0.089*	0.087*	0.068*	0.096*	0.082*	0.120*	0.094*	0.098*
11	0.678	0.074*	0.070*	0.115*	0.081*	0.066*	0.792	0.485	1.015	0.116*
12	0.085*	0.069*	0.097*	0.088*	0.101*	0.116*	0.123*	0.056*	0.068*	0.065*
15	0.062*	0.066*	0.601	0.043*	0.092*	0.053*	0.135*	0.069*	0.212	0.063*
27	0.220	0.730	0.518	0.090*	0.577	0.363	0.636	0.485	0.904	0.419
28	0.196	0.347	0.124*	0.076*	0.699	0.371	0.108*	0.087*	0.787	0.078*
34	0.107*	0.167*	0.121*	0.215	0.090*	0.091*	0.069*	0.131*	0.064*	0.069*
35	0.047*	0.047*	0.056*	0.042*	0.050*	0.046*	0.059*	0.052*	0.060*	0.046*
37	0.360	0.046*	0.780	0.033*	0.588	0.049*	0.063*	0.058*	0.052*	0 050*
38	0.049*	0 047*	0.050*	0 041*	0.055*	0.059*	0.063*	0.051*	0.054*	0 055*
40	0.078*	0 032*	0.035*	0.029*	0.063*	0.055*	0 054*	0.046*	0.055*	0.052*
62	0.052*	0.034*	0.051*	0.032*	0.063*	0.069*	0.052*	0.067*	0 059*	0.059*
85	0.731	0.39	0.575	0.004*	0.971	0.062*	0.059*	0.067*	0.041*	0 032*
87	0.058*	0.061*	0.051*	0.071*	0.076*	0.083*	0.053*	0.073*	0.097*	0 080*
95	0.069*	0.014*	0.045*	0.023*	0.043*	0.119*	0.06*	0.097*	0.023*	0.047*
106	0.061*	0.080*	0.151*	0.071*	0.316	0.047*	0.039*	0.064*	0.073*	0.040*
108	0.915	0.044*	0.865	0.781	0.663	0.037*	0.779	0.058*	0.518	0.078*
132	0.064*		0.073*	0.083*	0.062*		0.063*	0.060*	0.074*	0.081*
134	0.095*		0.087*	0.074*	0.095*	0.133*	0.123*	0.106*	0.073*	0.091*
140	0.051*	0.076*	0.072*	0 069*	0.077*	0.128*	0 119*	0.120*	0.105*	0.062*
144	0.062*	0.081*	0.055*	0.073*	0.077*	0.120*	0.172*	0.061*	0.079*	0.144*
145	0.077*	0.103*	0.082*	0.073*	0.068*	0.070*	0.066*	0.082*	0.097*	0.093*
149	0.139*	0.129*	0.108*	0.550	0 899	0.692	0.294	0.258	0.774	0.107*

* ELISA values which were classified as negative for TuMV-infection using a maximum threshold of negative values, four standard deviations above the mean of 8 Kordaat controls

Table 4b. ELISA values for recombinant F_3 families of a cross between Calmar and Cobbham Green. Ten individuals of each of 23 families were inoculated with TuMV and assayed for TuMV-infection by ELISA 6 weeks post-inoculation. Dashed lines indicate dead plants. Blanks are missing data.

F_2 parent	1	2	3	4	5	6	7	8	9	10
8	0.026*	0.043*	0.066*	0.065*	0.053*	0.051*	0.052*	0.051*	0.040*	0.051*
11	0.548	0.037*	0.043*	0.053*	0.034*	0.052*	1.009	0.845	1.378	0.690
12	0.045*	0.037*	0.049*	0.050*	0.051*	0.059*	0.083*	0.061*	0.044*	0.058
15	0.056*	0.049*	0.738	0.062*	0.078*	0.057*	0.852	0.039*	1.238	0.042*
27	0.630	0.748	0.955	0.058*	1.318	1.242	1.037	0.33	1.046	1.042*
28	0.468	0.567	0.839	0.039*	0.934*	0.608	0.206	0.074*	0.812	0.069*
34	0.049*	0.047*	0.054*	0.052*	0.052*	0.060*	0.068*	0.070*	0.044*	0.059*
35	0.725	0.053*	0.048*	0.063*	0.059*	0.063*	0.088*	0.148*	0.060*	0.060*
37	0.262	0.064*	0.888	0.064*	1.098	0.071*	0.089*	0.098*	0.081*	0.069*
38	0.069*	0.060*	0.068*	0.083*	0.055*	0.072*	0.086*	0.091*	0.066*	0.054*
40	0.077*	0.070*	0.070*	0.054*	0.085*	0.083*	0.096*	0.075*	0.092*	0.082*
62	0.070*	0.062*	0.051*	0.084*	0.078*	0.084*	0.079*	0.077*	0.085*	0.095*
85	1.248	0.093*	1.666	0.027*	1.488	0.104*	0.065*	0.573	0.053*	1.185
87	0.086*	0.053*	0.064*	0.076*	0.072*	0.061*	0.048*	0.047*	0.981	0.062*
95	0.070*	0.07*	0.074*	0.079*	0.085*	0.060*	0.064*	0.064*	0.049*	0.055*
106	0.064*	0.109*	1.324	0.092*	1.420	0.079*	0.072*	0.066*	0.071*	0.061*
108	1.165	0.086*	1.890	1.585	1.230	1.115	1.057	0.076*	1.251	1.371
132	0.081*		0.053*	0.074*	0.052*		0.056*	0.051*	0.051*	0.060*
134	0.058*		0.051*	0.054*	0.051*	0.044*	-----	0.056*	0.053*	0.048*
140	0.021*	0.035*	0.027*	0.030*	0.020*	0.033*	0.030*	0.025*	0.048*	0.045*
144	0.089*	0.089*	0.082*	0.072*	0.072*	0.067*	0.068*	0.076*	0.069*	0.064*
145	0.096*	0.079*	0.082*	0.093*	0.099*	0.089*	0.075*	0.093*	0.085*	0.082*
149	1.052	1.530	0.392	1.188	1.196	0.479	1.765	1.720	0.827	1.364

* ELISA values classified as negative for TuMV-infection using a maximum threshold of negative values, four standard deviations above the mean of 8 mock-inoculated controls.

Table 4c. ELISA values for recombinant F_3 families of a cross between Calmar and Cobbham Green. Ten individuals of each of 23 families were inoculated with TuMV and assayed for TuMV-infection by ELISA 12 weeks post-inoculation. Dashed lines indicate dead plants. Blanks are missing data.

F_2 Parent	1	2	3	4	5	6	7	8	9	10
8	0.063*	0.053*	0.042*	0.058*	0.039*	0.082*	0.083*	0.069*	0.034*	0.078*
11	-----	0.062*	0.069*	0.059*	0.051*	0.103*	-----	-----	-----	-----
12	0.040*	0.044*	0.037*	0.043*	0.038*	0.037*	0.045*	0.043*	0.027*	0.032*
15	0.049*	-----	-----	0.051*	-----	0.038*	-----	0.021*	-----	0.032*
27	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
28	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
34	0.085*	0.031*	0.056*	0.054*	0.063*	0.066*	0.036*	0.059*	0.037*	0.028*
35	-----	0.059*	0.019*	0.035*	0.040*	0.044*	0.044*	0.046*	0.038*	0.038*
37	-----	0.035*	-----	0.036*	-----	0.031*	0.034*	-----	0.037*	0.040*
38	0.038*	0.036*	0.045*	0.043*	0.045*	0.049*	0.060*	0.054*	0.053*	0.038*
40	0.081*	0.067*	0.076*	0.093*	0.088*	0.106*	0.086*	0.080*	0.087*	0.076*
62	0.054*	0.055*	0.044*	0.039*	0.066*	0.066*	0.054*	0.058*	0.047*	0.045*
85	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
87	0.053*	-----	0.052*	0.039*	0.054*	0.037*	0.061*	0.061*	-----	0.062*
95	0.044*	0.065*	0.053*	0.053*	0.051*	0.065*	0.058*	0.056*	0.057*	0.067*
106	0.028*	0.050*	-----	0.042*	-----	0.068*	0 088*	0 078*	0 082*	0.049*
108	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
132	0.032*		0.020*	-----	0.026*		0.028*	0.015*	0.038*	0.026*
134	0.050*		-----	0.037*	0.025*	0.031*	-----	-----	0.038*	0.056*
140	0.028*	-----	0.036*	0.021*	0.033*	0.037*	0.056*	-----	0.035*	0.031*
144	0.025*	0.033*	0.026*	0.025*	0.056*	-----	0.028*	0.044*	0.060*	0.031*
145	0.146*	0.090*	0.095*	0.040*	0.050*	0.035*	0.041*	-----	0.047*	0.028*
149	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

* ELISA values classified as negative for TuMV-infection using a maximum threshold of negative values, four standard deviations above the mean of 8 mock-inoculated controls.

into the phenotyping experiment. At least one positive reading by ELISA was required in order for a plant to be classed as susceptible to TuMV. Using a binomial distribution, it was determined that 10 F_3 individuals give $P_{(x=10)}=0.056$, or 94.6% confidence in the genotyping. Eighteen F_3 individuals give 99.4% confidence [$P_{(x=18)}=0.006$]. After combining the genotypes of the 23 F_2 recombinants with data provided by R. W. Michelmore for markers in the area of *Dm*5/8 (table 1), it was found that *Tu* was located between markers *OPM18* and *OPY13* (early map not shown).

The genotypes of a few families were in question due to either the death of some F_3 individuals or to contradictions between F_2 genotypes for *Tu* and the location of *Tu*. Eight individuals of these F_3 families (#8, #34, #35, #95, #134, #140, #144, and #145) were retested to determine the genotype at *Tu* for the F_2 progenitors (table 5a, 5b) and to increase the confidence in the genotyping from 94.6% to 99.4%. Some plants died early in the experiment, as indicated in the tables by blank spaces, and so the numbers of individuals in some F_3 families vary from the expected n=10 or n=18. Families were retested because it was unclear whether they were of genotype *TuTu* or *Tutu*. ELISA results from the total number of individuals surviving from both experiments were used to determine F_2 genotypes at *Tu* (table 6). All 23 recombinant F_2 genotypes for *Tu* (table 6) were combined with data provided by R. W. Michelmore for markers in the genomic area of *Dm*5/8 (table 1) for analysis using Mapmaker 3.0. *Tu* remained between OPM18 and OPY13 in an interval of 2.5 cM on the genetic map of *L. sativa* (figure 10).

An F_2 population of 66 individuals resulting from a cross between cv. Calmar and cv. Kordaat was used by Landry *et al.* (1987) to construct the genetic map of lettuce.

Table 5a. ELISA values for extended recombinant F_3 families of a cross between cvs. Calmar and Cobbham Green were inoculated with TuMV and assayed using ELISA 5 weeks post-inoculation.

F2	1	2	3	4	5	6	7	8
8	0.072*	0.051*	0.033*	0.040*	0.064*	0.076*	0.061*	0.060*
34	0.065*	0.161	0.075*	0.006*	0.031*	0.045*	0.160	0.134
35	1.696	0.060*	0.052*	0.025*	0.055*	0.039*	0.034*	0.029*
95	0.139	0.101*	0.096*	1.640	0.081*	0.067*	0.084*	0.095*
134	0.103*	0.128	0.071*	0.028*	0.089*	0.088*	0.033*	0.041*
140	0.027*	0.041*	0.024*	0.023*	0.154	0.130	0 104*	0 112
144	0.033*	0.037*	0.028*	0.026*	0.117	0.099*	0.155	0 128
145	0.056*	0.056*	0.043*	0.057*	0.091*	0.100*	0.035*	0.037*

* ELISA values classified as negative for TuMV-infection using a maximum threshold of negative values, four standard deviations above the mean of 8 mock-inoculated controls.

Table 5b. ELISA values for extended recombinant F3 families of a cross between cvs. Calmar and Cobbham Green were inoculated with TuMV and assayed using ELISA after 9 weeks. Dashed lines indicate dead plants.

F2	1	2	3	4	5	6	7	8
8	0 091*	0.097*	0.076*	0.068*	0.070*	0.131*	0.134*	0.095*
34	0.107*	0.095*	0.108*	0.006*	0.130*	0.101*	0.103*	0.126*
35	0.115*	-----	0.095*	0.118*	0.111*	0.126*	0.177*	0.128*
95	0.094*	0.064*	1.002	0.094*	0.134*	0.133*	0.152*	0.107*
134	0.130*	0.098*	0.098*	0.120*	0.132*	0.124*	0.129*	0.083*
140	0.089*	0.084*	0.096*	0.074*	0.097*	0.085*	0.153*	0.073*
144	0.115*	0.103*	0.096*	0.116*	0.147*	0.073*	0.122*	0.123*
145	0.076*	0.088*	0.108*	0.115*	0.133*	0.091*	0.106*	0.090*

* ELISA values classified as negative for TuMV-infection using a maximum threshold of negative values, four standard deviations above the mean for 8 mock-inoculated Cobbham Green controls.

Table 6. Genotypes for F_2 progenitors for *Tu* as determined using F_3 families of a cross between cvs. Calmar and Cobbham Green. Phenotypic ratios within F_3 families were used to establish genotypes at *Tu* for F_2 progenitors (see table 3a, 3b, and 3c). Some families were extended to distinguish between *TuTu* and *Tutu* F_2 genotypes (see table 4a and 4b). Homozygous resistant genotypes (*TuTu*) are represented by B, susceptible by A (*tutu*), and heterozygotes (*Tutu*) by H.

F_2	Resistant	Susceptible	Genotype
8	10	0	B
11	5	5	H
12	10	0	B
15	5	5	H
27	0	10	A
28	0	10	A
34	18	0	B
35	16	2	H
37	6	4	H
38	10	0	B
40	10	0	B
62	10	0	B
85	0	10	A
87	8	2	H
95	17	1	H
106	8	2	H
108	0	10	A
132	7	1	H
134	14	0	B
140	16	0	B
144	16	0	B
145	16	0	B
149	0	10	A

Cultivars Kordaat and Cobbham Green are resistant to TuMV. These cultivars are of the butterhead type and may share loci other than *Tu*, although this has not been determined. RAPD markers found using bulks created from a cross between cvs. Calmar and Kordaat (section IV.4) were mapped using a cross between cvs. Calmar and Cobbham Green. Only five of these RAPD markers (*UBC346*, *UBC517*, *UBC563*, *UBC599*, and *UBC675*) are in both crosses and could be mapped. Small differences in the genomes of cvs. Kordaat and Cobbham Green may change the map distances between markers [i.e. map distances for markers on the cvs. Calmar x Cobbham Green map (section IV.4) may differ from those on the cvs. Calmar x Kordaat genetic map (Kesseli *et al.*, 1991, unpublished)]. Some marker orders could not be resolved (*OPM18*, *OPL08* and *OPY13*, *UBC517*, *UBC599*) using the mapping population of cvs. Calmar x Cobbham Green (142 F_2 individuals) (section IV.4).

A mapping population produced using cvs. Calmar and Kordaat of 203 F_2 individuals was obtained from R. W. Michelmore. F_3 families for nine F_2 recombinants in the area of *Tu* from this population will be used to check the genetic distances of new RAPD markers relative to the published map created using a cvs. Calmar x Kordaat cross. The cvs. Calmar x Kordaat cross contains 406 meioses verses 284 meioses in the cvs. Calmar x Cobbham Green cross. Markers *OPM18*, *OPL08* and *OPY13*, *UBC517*, *UBC599* may be resolved using this mapping population.

Eighteen F_3 individuals from each of nine progenitor F_2 plants resulting from a cross between cvs. Calmar and Kordaat were inoculated with TuMV at both the 3- and 5-leaf stages using 0.25 g/ml of TuMV-infected *B. perviridis* in KPO_4 buffer. DNA

representative of the F_2 parents was obtained by combining equal amounts of each F_3 plant in a family and extracting as described. Using F_3 phenotypes (table 7), F_2 genotypes at *Tu* (table 8) were determined. These genotypes, when visually compared to mapping information for RAPD markers in the area of *Tu* and *Dm*5/8, place *Tu* between *OPD08* and *OPU16* on the genetic map of *L. sativa* (table 9). Individuals #31 and #142 were the most informative for the location of *Tu* using this cross. The F_2 DNA of all nine plants will be used to perform RAPD-PCR using RAPD markers in the area of *Tu*. Segregation of the markers in the population will be mapped relative to *Tu* to determine genetic distances on the cvs. Calmar x Kordaat map.

4. Detection of RAPD-markers linked to Tu

Simultaneously, bulked segregant analysis (Michelmore *et al.*, 1991) was used to screen for additional RAPD markers linked to *Tu*. Pools of DNA from F_2 plants homozygous resistant (bulk A) or susceptible (bulk B) at the *Dm5/8* locus were combined to form bulks of DNA for each of the two phenotypes. Allelic variations between individuals are homogenized in the pools, leaving the presence or the absence of the resistance locus as the remaining genetic difference. The two pools were used as substrate for RAPD-PCR analysis. Pools were made on the basis of the *Dm5/8* phenotype, and not *Tu*, by R. W. Michelmore since the two loci are linked and a population segregating for *Dm5/8* was available from the work of Kesseli *et al.* (unpublished). Homozygosity of F_2 individuals at the *Dm5/8* locus had been determined by testing F_3 families for downy mildew resistance. The F_2 population resulted from a

Table 7. ELISA values for 9 F_3 families from a cross between Calmar and Kordaat. All plants were inoculated with TuMV and assayed by ELISA 5 weeks post-inoculation. Blanks indicate missing data.

F3	31	118	129	131	135	142	201	220	240
1	0.060*	1.032	0.228	0.070*	-0.044*	0.233	-0.013*	0.362	1.136
2	0.041*		0.143	0.191	-0.028*	0.630	*****	0.275	1.407
3	1.333	0.492	0.035*	-0.018*	1.077	-0.007*	0.573	0.330	1.287
4	0.006*	0.119*	0.883	-0.036*	-0.046*	-0.017*	0.665	0.862	0.716
5	0.134	0.116*	0.092*	-0.001*	0.143	-0.011*	1.050	0.927	1.784
6	0.154		0.296	-0.008*	0.055*	-0.019*	1.956	1.300	-0.079*
7	0.065*	0.955	0.197	0.165	0.061*	-0.003*	*****	0.181	0.648
8	0.009*		0.102*	0.146	0.510	-0.043*	*****	1.414	0.959
9	0.086*	1.086	0.125	-0.005*	0.075	-0.020*	1.838	0.795	0.275
10	0.069*	0.796	0.177	-0.019*	0.100	1.062	1.182	*****	0.365
11	0.071*	0.677	0.182	0.045*	-0.027*	0.057*	1.715	0.239	1.507
12	0.038*	0.511	0.786	0.484	-0.048*	0.026*	1.176	-0.090*	-0.098*
13	0.031*	0.790	0.101*	0.033*	0.975	-0.010*	1.472	0.154	0.461
14	0.032*	0.681	1.039	0.700	1.044	0.003*	-0.074*	0 584	-0.092*
15	0.084*	0.811	0.147	0.083	0.062*	0.043*	1.164	0.385	1.061
16	0.074*	0.243	0.595	0.129	0.099	-0.037*	0.693	1.899	1.287
17	0.074*	0.742	0.121	0.046*	0.086	0.016*	1.608	0.360	1.184
18	0.068*	1.249	0.160	0.008*	0.038*	0.058*	0.052	1.662	0.477

* ELISA values classified as negative for TuMV-infection from a maximum threshold of negative values, four standard deviations above the mean of 8 mock inoculated controls.
***** ELISA values greater than 2.000.

Table 8. Genotypes for F_2 progenitors for *Tu* as determined using F_3 families of a cross between cvs. Calmar and Kordaat. Phenotypic ratios within F_3 families were used to establish the parental genotypes (see table 7). Homozygous resistant genotypes (*TuTu*) are represented by B, susceptible (*tutu*) by A, and heterozygoyes (*Tutu*) by H.

F_2	Resistant	Susceptible	Genotype
31	17	1	H
118	0	15	A
129	9	9	H
131	11	7	H
135	9	9	H
142	15	3	H
201	0	16	A
220	0	17	A
240	0	15	A

Table 9. Genotypes for F_2 recombinants from a cross between cvs. Calmar and Kordaat for *Tu* relative to markers in the area of *Dm5/8*. Mapping information for markers in the area of *Dm5/8* for nine recombinant F_2 individuals were provided by R. W. Michelmore and combined with genotypes at *Tu* found by phenotyping 18 F_3 plants from each of the nine F_2 progentors. Letters in the table represent genotypes A = homozygous Calmar, B = homozygous Kordaat, H = heterozygote, C = heterozygote or B, D = heterozygote or A. Dashed lines represent missing data.

F_2	*OPC08*	*OPS12*	*OPX03*	*OPH15*	*Dm5/8*	*OPD08*	*OPF12*	*Tu*	*OPY13*	*OPU16*
31	-	-	C	-	H	D	D	H	D	A
118	A	D	A	-	A	D	D	A	D	C
129	-	-	C	-	H	D	D	H	D	A
131	-	-	A	-	A	-	-	H	D	C
135	-	-	A	-	H/A	-	D	H	D	C
142	-	B	C	B	B	B	D	H	D	-
201	-	-	C	-	H	D	D	A	D	A
220	-	-	C	-	H	D	D	A	D	A
240	-	-	C	-	H	D	D	A	D	A

cross between cvs. Kordaat (*TuTu*) and Calmar (*tutu*). DNA extracted from 12 F_3 plants was used by R. W. Michelmore to create each pool.

Two-hundred and sixty-five primers of ten bp were screened using the bulks previously produced in the region of *Dm*5/8 (Michelmore *et al.*, 1991), and seven polymorphic markers (*UBC346, UBC517, UBC563, UBC566, UBC599, UBC675, UBC688*) were identified (figure 6, table 10). Polymorphisms detected between the two bulks were examined in the F_2 population for segregation with the *Tu* locus. All of these new RAPD markers were dominant, i.e. either the polymorphism was present or absent, with no length polymorphisms to distinguish between heterozygotes and homozygotes. If the polymorphism was present in the resistant (B) bulk, then all F_2 individuals displaying the polymorphic band were scored as C (either homozygote, BB, or heterozygote, BA) and those F_2 individuals which did not have the band were scored as A. If the polymorphism was present in the susceptible (A) bulk, then all F_2 individuals with the polymorphic band were scored as D (either homozygote, AA, or heterozygote, BA) and F_2 individuals without the polymorphism were scored as B. The mapping population of 142 individuals at loci scored by R. W. Michelmore in the area of *Dm*5/8 was used to produce the Mapmaker file (table 1) in combination with data for new RAPD markers (table 11).

The polymorphic band was faint for some primers, making it difficult to determine whether the band was absent, or simply low in intensity (figure 7). The RAPD marker data for F_2 individuals recombining in the vicinity of the *Tu* locus were verified by Southern blotting to avoid mis-scoring. RAPD amplification products were

Figure 6. Identification of RAPDs linked to the *Tu* locus by bulked segregant analysis. Random 10 bp primers were used to amplify DNA from homozygous resistant or susceptible pools of F_2 individuals. The pools were formed on the basis of the phenotype at the *Dm5/8* locus, which is linked to *Tu*. The F_2 individuals were from a cross between cvs. Calmar and Kordaat. Each pair of lanes (identified by numbers) are amplification products from one primer, using two pools (A and B) of DNA. Primers used, in order from 1 to 7, are UBC346, UBC349, UBC506, UBC517, UBC566, UBC578, UBC599. Amplification products were separated by agarose gel electrophoresis.

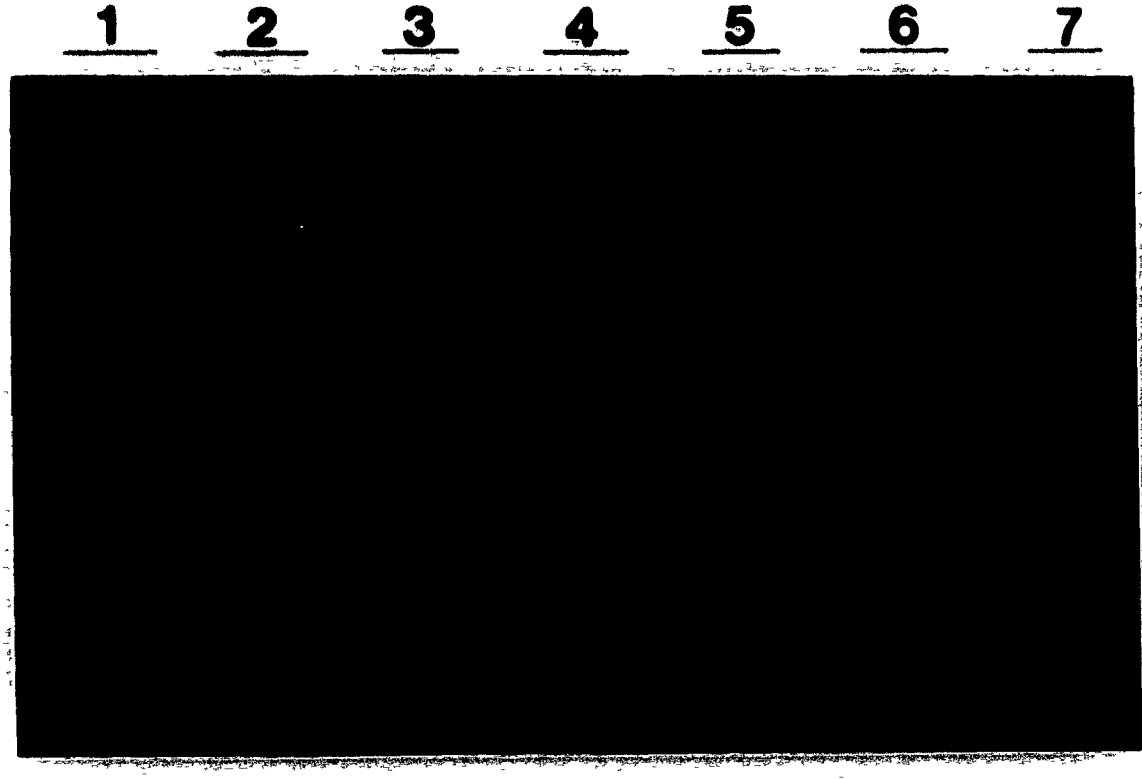

1
2
3
4
5
6
7

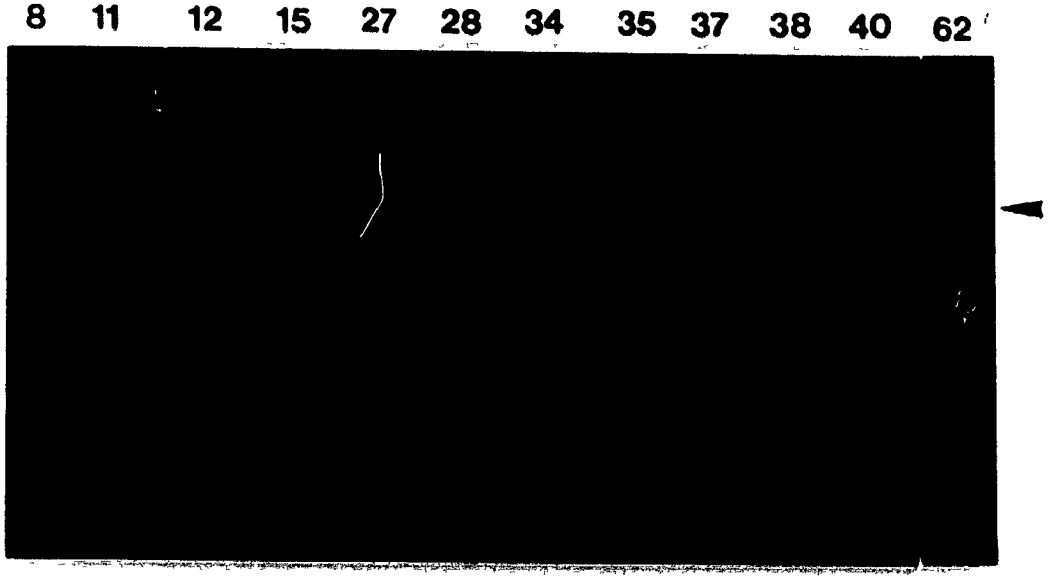
Table 10. Primers obtained from J.E. Carlson, University of British Columbia (UBC), which displayed polymorphism between bulks created for the *Dm5/8* locus and were found linked to *Tu*. Polymorphic bands from the TuMV-susceptible (Calmar) bulk are designated A and those from the resistant bulk (Kordaat) are B. All polymorphisms segregated as dominant loci. Sequences are in the 5' to 3' orientation.

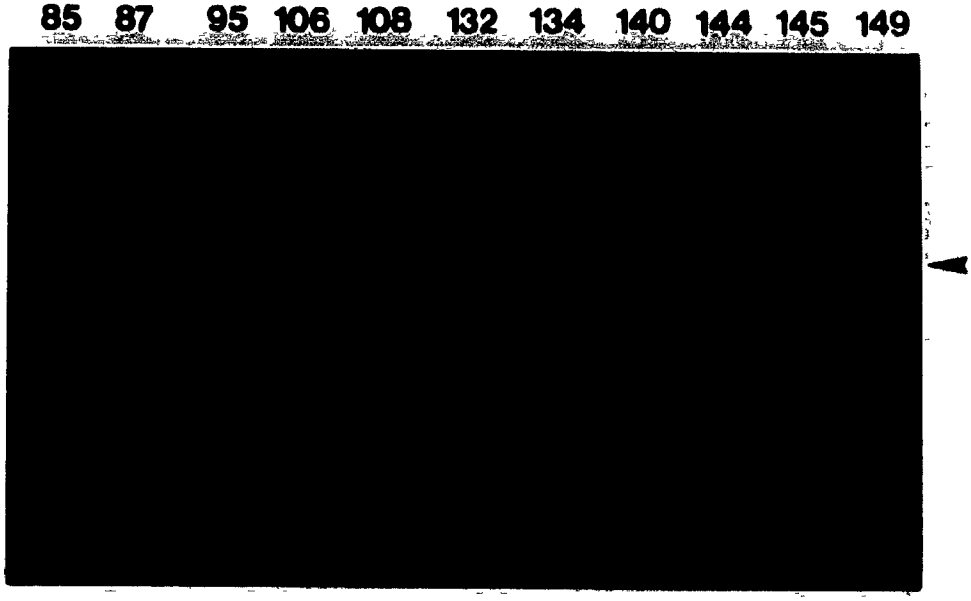
Primer #	polymorphism	band size	sequence
UBC346	B	1065	TAGGCGAACG
UBC517	A	1395	GGTCGCAGCT
UBC563	B	710	CGCCGCTCCT
UBC566	A	735	CCACATGCGA
UBC599	A	2480	CAAGAACCGC
UBC675	B	1370	ACCGGTGGAG
UBC688	A	910	GCAGGAGCGT

Table 11. Genotypes for F_2 recombinant individuals from a cross between cvs. Calmar (TuMV-susceptible) and Cobbham Green (TuMV-resistant) for the five new RAPD markers, *UBC346*, *UBC517*, *UBC563*, *UBC599*, and *UBC675* relative to *Tu*. Numbers in the left column represent F_2 individuals. Letters in the table represent genotypes A = homozygous Calmar, B = homozygous Cobbham Green, H = heterozygote, C = heterozygote or B, D = heterozygote or A. Genotypes were obtained by scoring bands on a gel and were verified using Southern blotting and probing with the polymorphic band obtained by bulked segregant analysis. Dashed lines represent missing data.

F_2	$UBC675_{1370}$	$UBC563_{710}$	*Tu*	$UBC346_{1065}$	$UBC517_{1395}$	$UBC599_{2480}$
8	C	C	B	C	D	D
11	A	C	H	C	D	D
12	A	C	B	C	B	B
15	A	-	H	C	D	D
27	C	A	A	A	D	D
28	C	A	A	A	D	D
34	C	C	B	C	B	B
35	C	C	H	C	B	B
37	A	C	H	C	D	D
38	A	A	B	C	B	B
40	A	-	B	C	B	B
62	C	C	B	C	B	B
85	A	A	A	C	D	D
87	A	A	H	C	D	D
95	A	C	H	C	D	D
106	C	C	H	C	B	B
108	C	A	A	A	D	D
132	C	C	H	C	D	D
134	C	C	B	C	B	B
140	A	C	B	C	B	B
144	A	-	B	C	B	B
145	A	C	B	C	B	B
149	C	C	A	A	D	D

Figure 7. Agarose gel electrophoresis of RAPD amplification products using primer *UBC675* and F_2 recombinants. The population from which the 23 recombinants in the *Dm5/8* region were derived is segregating for *Tu*. The F_2 population from a cross between cvs. Calmar and Cobbham Green, and were previously identified as recombinants in the *Dm5/8* region using RFLP markers. The genotype for the *Tu* locus was determined by inoculation with TuMV of F_3 individuals resulting from the selfing of F_2 parents. The genotype corresponding to each F_2 individual is shown in Table 1. The gels were stained with ethidium bromide and the DNA visualized using an ultraviolet transilluminator. An arrow indicates the polymorphism which is segregating in this population.


8
11
12
15
27
28
34
35
37
38
40
62


85
87
95
106
108
132
134
140
144
145
149

transferred from the gel to a nylon membrane for probing. The polymorphic band for each of five (*UBC346, UBC599, UBC517, UBC675, OPM18*) primers was excised from the gel, labelled, and used as a probe on a Southern blot of amplification products from the same primer. For marker *UBC675*, individuals #27 and #35 were scored as A (no band) from examination of the gel (figure 7). Using the polymorphic band generated by primer UBC675 to probe RAPD-PCR products, it was found that #27 and #35 were C (have the band) (figure 8). This change in the data changed *UBC675* from unmappable to 8.9 cM from *OPC08* (figure 10).

RAPD marker data and the genotypic information for *Tu* for all 23 recombinant F_2 individuals in the region were analyzed for linkage using Mapmaker 3.0 (Lander *et al.*, 1987) (table 1 and 11). Five markers, *UBC346*, *UBC517*, *UBC563*, *UBC599*, and *UBC675* detecting a DNA polymorphism between the resistant and the susceptible bulks were confirmed linked to the *Tu* locus (figure 10). The order of markers *OPY13, UBC517* and *UBC599* and markers *OPM18* and *OPL08* could not be resolved using a population of 142 F_2 individuals. Two markers, *UBC566* and *UBC688*, did not segregate in the mapping population used, indicating that the polymorphism identified in the bulks (produced from a cross of cv. Calmar and cv. Kordaat) was absent in the parents used to generate the mapping population (cv. Calmar x cv. Cobbham Green). *UBC566* and *UBC688* need to be examined in a segregating population produced using cv. Calmar and cv. Kordaat as parents.

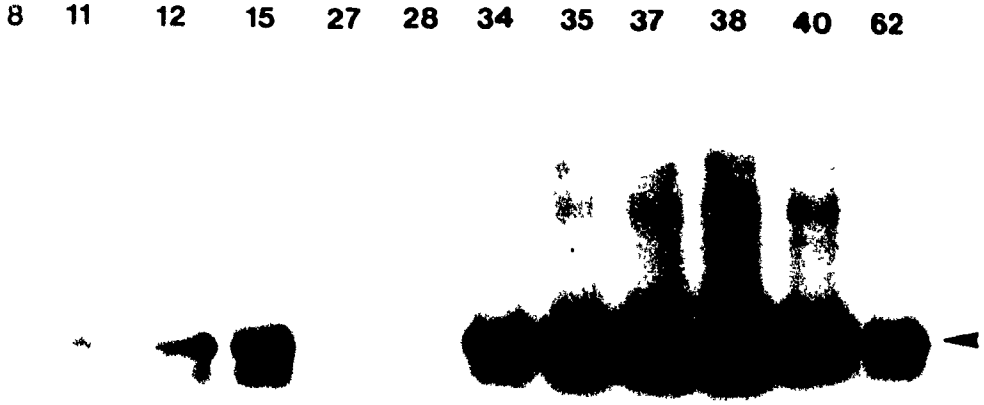
There are discrepancies between the genetic distance between *UBC346* and Tu determined using Mapmaker 3.0 and visual examination of the data for *UBC346*

Figure 8. Southern blot of RAPD amplification of F_2 recombinants with primer *UBC675* and probe *UBC675*. A nonradioactively labeled probe was prepared for primer *UBC675* by labeling the polymorphic band observed in bulked segregant analysis and used on a Southern transfer of RAPD amplification products (using primer *UBC675*) from F_2 individuals recombining in the region of *Dm*5/8. An arrow indicates the polymorphic band found using primer *UBC675* which is segregating in the population.


8 11 12 15 27 28 34 35 37 38 40 62


85 87 95 106 108 132 134 140 144 145 149

Figure 9. Southern blot of RAPD amplification of F_2 recombinants with primer *UBC346* and probe *UBC346*. A radioactively labeled probe was prepared for primer *UBC346* by excising and labeling the polymorphic band observed in bulked segregant analysis and used on a Southern transfer of RAPD amplification products (from primer *UBC346*) of 23 F_2 recombinants in the area of *Dm*5/8.


8 11 12 15 27 28 34 35 37 38 40 62

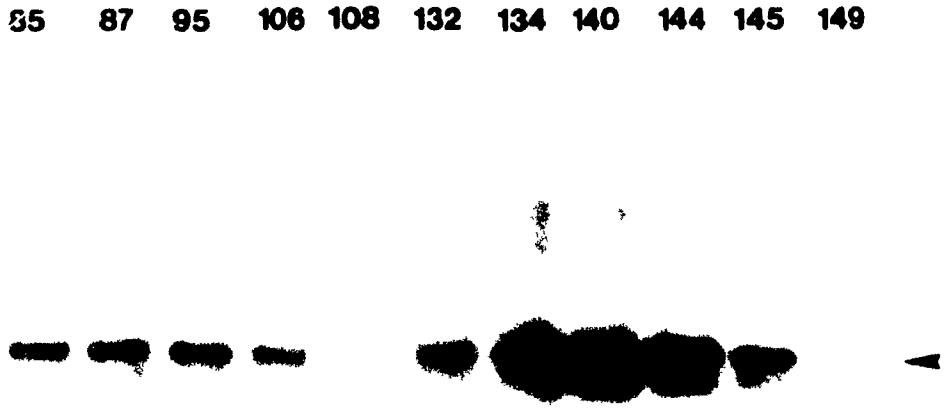
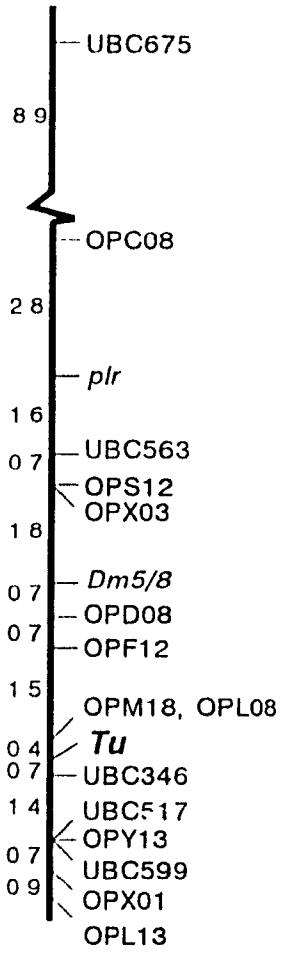

85 87 95 106 108 132 134 140 144 145 149

Figure 10. Genetic map of *L. sativa* at the *Tu* locus. Genetic map of *L. sativa* surrounding the *Tu* locus. The mapping population consisted of 142 F_2 individuals from a cross between cultivars Calmar and Cobbham Green. Genetic distances between loci are shown in centiMorgans. All the markers are RAPD markers, except for *Dm5/8* (resistance to *Bremia lactucae*), *plr* (resistance to *Plasmopara lactucae radicis*), and *Tu*.


UBC675
8 9
OPC08
2 8
plr
1 6
0 7
UBC563
OPS12
OPX03
1 8
0 7
Dm5/8
OPD08
0 7
OPF12
1 5
OPM18, OPL08
0 4
Tu
0 7
UBC346
1 4
UBC517
OPY13
0 7
UBC599
0 9
OPX01
OPL13

compared to surrounding loci. According to a visual examination of the data, there is one recombination event between *Tu* and *UBC346* in individual #85 (table 11). Mapmaker, when performing three-point and multi-point analysis on a combination of data from R. W. Michelmore (table 1) and the new RAPD markers and *Tu* (table 11), puts *UBC346* 0.7 cM away from *Tu* (figure 10).

Genetic distance is a function of recombination frequency where 1 cM between two markers is equal to 1 recombination event between those markers in a population of 100 individuals. A distance of 0.7 cM between two markers from a population of 142 individuals is equal to two recombination events (0.35 cM/recombination). When the triple-error detection of Mapmaker 3.0 is used, an error in the data is pointed out in individual #85 between *OPL08*, *Tu*, and *UBC346*. Mapmaker gives an output of H-A-H for these markers, respectively, in individual #85, showing that a double recombination event had to have taken place to reconcile the position of *UBC346*. Double recombination events are rare and so Mapmaker 3.0 points out these points as potential errors in the data. Mapmaker is reading the D and C at loci *OPL08* and *UBC346* as H and indicates that the genotype of #85 may be wrong at *Tu*. Instead of *tutu*, #85 may be *Tutu*. Using error detection, Mapmaker 3.0 gives a distance between *Tu* and *UBC346* as 0 cM.

To determine whether there is an error in genotyping, 18 F_3 plants of #85 should be rephenotyped for TuMV-susceptibility or resistance. Alternatively, there may be a flaw in the Mapmaker program which fails to recognize that, using dominant markers, #85 could be of genotype AA on the *Dm*5/8 side of *Tu* instead of AB (which Mapmaker

assumes) (table 1). Individual #85 could be AB on the *UBC346* side of *Tu*, making a single recombination event between *Tu* and *UBC346*, and solving the dilemma. If this is the case, the genetic distance on the map (figure 10) is 0.4 cM instead of 0.7 cM. The genetic distance between *Tu* and *UBC346* must be resolved using the cv. Calmar x cv. Kordaat cross (section IV.3).

Three of five new RAPD markers (*UBC346*, *UBC517*, and *UBC599*) were clustered in a 1.4 cM region near *Tu* (figure 10). Although the markers identified by bulked segregant analysis were not distributed randomly, all polymorphic markers identified mapped to a region spanning 20 cM around *Dm5/8*. This is in contrast to the results obtained from screening near isogenic lines by Paran *et al.* (1991) where only half of the polymorphisms obtained were linked to the target locus. Half of the polymorphisms segregated randomly and appeared to be linked to genomic fragments from the donor parent that were not selected for during the backcrosses (Paran *et al.*, 1991).

The closest markers flanking the *Tu* gene are OPM18 and UBC346 at 0.4 and 0.7 cM respectively (figure 10). It is likely that many more markers will be required before map-based cloning becomes feasible. However, there is a wide variation in the relationship between genetic and physical distances, from approximately 14 kbp/cM for the *Bz* locus in corn (Dooner, 1986) to over 4 Mbp/cm around the *Tm-2a* gene in tomato, conferring resistance to Tobacco Mosaic Virus. The *Tm-2a* gene is located in a centromeric region. The physical distance separating the gene and the marker is thus larger than the genetic distance would suggest. Tanksley *et al.* (1992) have determined

that *Tm-2a* is separated from the marker by at least 2 x 10^6 bp. This is in sharp contrast to the average (for the whole tomato genome) of approximately 500,000 bp per cM (Young *et al.*, 1988). Other viral resistance genes have been mapped, but linkage in most cases is more distant (Timmerman *et al.*, 1993).

The linkage detected here between the resistance gene and the markers is sufficiently close to warrant examination of the physical distance separating the two markers and the *Tu* gene. The distance between the two markers will in fact represent the maximum distance that will need to be covered by clones in order to recover a cloned copy of the *Tu* gene.

Larger segregating populations must be screened for rare recombinants in the *Tu* region that will prove useful for mapping in the eventuality the physical distance is too large to recover in individual clones, including yeast artificial chromosome clones. Concurrently, new markers must be sought using the bulks created for the *Tu* locus.

To aid future screening for markers linked to *Tu*, an F_2 population from a cross between cv. Calmar (susceptible to TuMV) and cv. Kordaat (resistant) which segregated for *Tu* was obtained from Dr. M.G. Fortin. Ten individuals per bulk are sufficient to ensure that all polymorphisms detected between bulks will be linked to the target locus (Michelmore *et al.*, 1991). To be able to find polymorphisms in *cis* and in *trans* to *Tu*, 80 F_2 plants were selfed and 18 F_3 individuals (F_3 family) from each F_2 parent were inoculated with TuMV at both the 3- and 5-leaf stages for phenotyping for resistance or susceptibility by ELISA. With a segregation ratio for *Tu* of 1(*TuTu*):2(*Tutu*):1(*tutu*), and assuming that some of the F_2 individuals will not attain reproductive maturity, eighty F_2

individuals were thought to be sufficient to enable the production of bulks containing at least 10 individuals each for the *Tu* locus. Sixty-five F_2 plants survived and produced seed. Only one family produced seed which was not viable. From an F_2 population of 65, 16 individuals should be homozygous for *Tu* and 16 for *tu*. While the F_2 plants were growing, leaf material was collected from all plants and stored at -70°C. DNA from F_2 plants found to be *tutu* (from leaf at -70°C) will be combined in one bulk (bulk A) and DNA from those found to be *TuTu* will be combined to form bulk B and screened for polymorphism linked to *Tu* using RAPDs. Eleven F_2 individuals for each class (*TuTu* and *tutu*) were unambiguously identified using the ELISA values from respective F_3 families (R. Singh, unpublished). This is sufficient to ensure that polymorphisms detected in future RAPD screening of the bulks will be linked, either in *cis* or in *trans*, to the *Tu* locus.

The production of bulks for the *Tu* locus was important as, if markers found using the bulks for *Dm*5/8 created by R. W. Michelmore are not close in physical terms for chromosome walking, new markers closer to *Tu* will be necessary.

V. Conclusion

The location of *Tu* on the genetic map of *L. sativa* is the first step towards the eventual cloning and characterization of this viral resistance gene.

Since the work of Duffus and Zink (1970) it has been known that *Tu* is linked to *Dm*5/8. Studies at U. of C., Davis by R. W. Michelmore for the characterization of the *Dm*5/8 locus provided material such as recombinants in the area of *Dm*5/8 and bulks for *Dm*5/8 which were useful for orienting *Tu* relative to *Dm*5/8 and finding new markers linked to *Tu*.

Conditions for inoculation using TuMV were optimized before performing the phenotyping experiments (section IV.1). An antibody to the TuMV isolate being used was produced to recombinant TuMV-CP expressed in *E. coli* for use in ELISA (section IV.2). Genotypes at *Tu* for F_2 individuals recombining in the area of *Dm*5/8 (obtained from R. W. Michelmore) were found by phenotyping F_3 families of each F_2 progenitor for resistance or susceptibility to TuMV using ELISA (section IV.3). With these genotypes, *Tu* was located between two genetic markers, *OPM18* and *OPY13*, on the genetic map of *L. sativa*. The distance between *Tu* and *OPM18* was 0.4 cM.

To resolve markers *OPM18*, *OPL08* and *UBC517, UBC599, OPY13* which segregate as two loci in the cv. Calmar x cv. Cobbham Green mapping population of 142 individuals, another population containing 203 individuals was obtained from R. W. Michelmore. Eighteen F_3 offspring for each of nine F_2 individuals from a cv. Calmar x cv. Kordaat cross recombining in the area of *Tu* were phenotyped for resistance or susceptibility to TuMV by ELISA. F_2 genotypes were obtained using F_3 phenotypic

ratios. Using the F_2 genotypes at *Tu* and mapping information provided by R. W. Michelmore, it was found that *Tu* is located between markers *OPF12* and *OPY13* (table 8).

Five new RAPD markers, *UBC346*, *UBC517*, *UBC563*, *UBC599*, and *UBC675* were found linked to *Tu* by bulked segregant analysis using bulks for the *Dm5/8* locus obtained from R. W. Michelmore (section IV.4). One marker, *UBC346*, was 0.7 cM from *Tu*. Two markers, *UBC566* and *UBC688*, which did not segregate in the cv. Calmar x cv. Cobbham Green mapping population (see above), will be examined for linkage to *Tu* in the cv. Calmar x cv. Kordaat cross using DNA extracted from pools of each F_3 family resulting from the nine informative F_2 recombinants.

Although two markers were closely linked in genetic terms to *Tu*, *OPM18/OPL08* at 0.4 cM and *UBC346* at 0.7 cM, it was possible that the physical distance between the markers was too large in bp for chromosome walking and jumping. The physical distance between these markers will have to be determined using pulsed-field gel electrophoresis (PFGE) and SCARs of *UBC346* and *OPM18/OPL08*. Markers more tightly linked to *Tu* may be necessary.

For the production of new bulks for the *Tu* locus, a F_2 population of 80 individuals segregating for *Tu* (created from a cross between cv. Calmar and cv. Kordaat) was obtained from M. G. Fortin and selfed. Seed was harvested and 18 F_3 individuals inoculated twice (at 3- and 5-leaf stage) with TuMV. An ELISA was performed by R. Singh (unpublished) and bulks will soon be produced for use in screening for new markers linked to *Tu*.

VI. References

Abel, P. Powell, Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T., and Beachy, R.N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Abo El-Nil, M.M., Zettler, F.W., and Hiebert, E. 1977. Purification, serology and some physical properties of dasheen mosaic virus. Etiology 67:1445-1450.

Adams, L.D. 1989. Two dimensional gel electrophoresis using the ISO-DALT system. in *Current Protocols in Molecular Biology*. F. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, G. Seidman, J.A. Smith, and K. Struhl. eds. Wiley.

Allison, R.F., Sorenson, J.C., Kelly, M.E., Armstrong, F.B., and Dougherty, W.D. 1985. Sequence determination of the capsid protein gene and flanking regions of tobacco etch virus: Evidence for synthesis and processing of a polyprotein in potyvirus genome expression. Proc. Natl. Acad. Sci. USA 82:3969-3972.

Arumuganathan, K., and Earle, E.D. 1991. Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol. Biol. Rep. 9:229-241.

Beckmann, J.S., and Soller, M. 1983. Restriction fragment length polymorphisms in genetic improvement: methodologies, mapping and costs. Theor. Appl. Genet. 67:35:43.

Bol, J.F. 1988. Structure and expression of plant genes encoding pathogenesis-related proteins. In *Plant gene research: Temporal and spatial regulation of plant genes*, Verma D.P.S., and Goldberg, R., ed., Springer-Verlag, Berlin. pp. 201-221.

Botstein, D., White, R., Skolnick, M., Davis, R. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Bowles, D.J. 1990. Defense-related proteins in higher plants. Annu. Rev. Biochem. 59:873-907.

Bruening, G., Ponz, F., Glascock, C, Russell, M.L., Rowani, A., Chay, C. 1987. Resistance of cowpeas to cowpea mosaic virus and to tobacco ringspot virus. in *Plant Resistance to Viruses*. Chichester,New York: Wiley. pp. 23-37.

Calder, V.L., and Palukaitis, P. 1992. Nucleotide sequence analysis of the movement genes of resistance breaking strains of tomato mosaic virus. J. Gen. Virol. 73:165-168.

Carr, J.P., and Klessig, D.F. 1989. The pathogenesis-related proteins of plants. In *Genetic engineering; principles and methods*, 11, Setlow J.K., ed., Plenum Press, New York. pp. 65-109.

Chaparro, J., Wilcox, P., Grattapaglia, D., O'Malley, D., McCord, S., Sederoff, R., McIntyre, L., and Whetten, R. 1992. Genetic mapping of pine using RAPD markers: Construction of a 191 marker map and development of half-sib genetic analysis. Advances in gene technology: Feeding the world in the 21st century Miami Winter Symp., Miami, Fla.

Choi, J.K., Maeda, T., and Wakimoto, S. 1977. An improved method for purification of turnip mosaic virus. Ann. Phytopath. Soc. Japan 43:440-448.

Choi, J.K., and Wakimoto, S. 1979. Characterization of the protein components of the turnip mosaic virus. Ann. Phytopath. Soc. Japan 45:32-39.

Clarke, M.F., Lister, R.M., and Bar-Joseph, M. 1986. ELISA techniques. Methods in Enzym. 118:742-766.

Coe, E.H., Hoisington, D.A., Neuffer, M.G. 1990. Linkage map of corn (maize) (*Zea mays* L.). In: O'Brien SJ (ed) Genetic maps, 5th edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor/New York.

Culver, J.N., Linkbeck, A.G.C., and Dawson, W.O. 1991. Virus-host interactions: Induction of chlorotic and necrotic responses in plants by tobamoviruses. Annu. Rev. Phytopathol. 29:193-217.

Dooner, H.K. 1986. Genetic fine structure of the bronze locus in maize. Genetics 113:1021-1036.

Doucet, R., Shattuck, V.I., and Stobbs, L.W. 1990. Rutabaga germplasm TuMV-R possessing resistance to Turnip Mosaic Virus. HortScience 25:583-584.

Doyle, J.J. and Doyle, J.L. 1987. Isolation of plant DNA from fresh tissue. Phytochem. Bull. 19:11.

Duffus, J., and Zink, F.W. 1969. A diagnostic host reaction for the identification of turnip mosaic virus. Plant Dis. Reporter 53:916-917.

Ellis, J.G., Lawrence, G.J., Peacock, W.J., and Pryor, A.J. 1988. Approaches to cloning plant genes conferring resistance to fungal pathogens. Ann. Rev. Phytopath. 26:245-263.

Feinberg, A.P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction

endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Flor, H.H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9:275-296.

Fraser, R.S.S. 1982. Biochemical aspects of plant resistance to virus disease: a review. Acta Horticulturae 127:101-116.

Fraser, R.S.S. 1983. Varying effectiveness of the *N'* gene for resistance to tobacco mosaic virus in tobacco infected with virus strains differing in coa. protein properties. Physiol. Plant Path. 22:109-119.

Fraser, R.S.S. 1987. Genetics of plant resistance to viruses. In *Plant Resistance to Viruses*. Wiley: Chichester. pp. 6-22.

Fraser, R.S.S. 1990. The genetics of resistance to plant viruses. Annu. Rev. Phytopathol. 28:179-200.

Gallagher, S., Winston, S.E., Fuller, S.A., and Hurrell, J.G.R. 1989. Immunoblotting and Immunodetection. In *Current Protocols in Molecular Biology*. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Sedman, J.A. Smith, and K. Struhl eds. Wiley.

Ganal, M.W., Young, N.D., and Tanksley, S.D. 1989. Pulsed field gelelectrophoresis and physical mapping of large DNA fragments in the *TM-2a* region of chromosome 9 in tomato. Mol. Gen. Genet. 215:395-400.

Gebhardt, C., Mugniery, D., Ritter, E., Salamini, F., and Bonnel, E. 1993. Identification of RFLP markers closely linked to the *H1* gene conferring resistance to *Globodera rostochiensis* in potato. Theor. Appl. Genet. 85:541-544.

Gianinazzi, S., Martin, C., and Vallée, J.C. 1970. Hypersensibilité auz virus, température et protéines solubles chez le *Nicotiana* Xanthi nc. Apparition de nouvelles macromolécules lors de la répression de la synthèse virale. C. R. Acad. Sci. Paris 270: 2383-2386.

Gianinazzi, S., and Kassanis, B. 1974. Virus resistance induced by polyacrylic acid. J. Gen. Virol. 23:1

Gianinazzi, S., Pratt, H.M., Shewry, P.R., and Miflin, B.J. 1977. Partial purification and preliminary characterization of soluble leaf proteins specific to virus infected tobacco plants. J. Gen. Virol. 34:345-351.

Green, E.D., and Olson, M.V. 1990. Systematic screening of yeast artificial-

chromosome libraries by use of the polymerase chain reaction. Proc. Natl. Acad. Sci. USA 87:1213-1217.

Harlow, E., and Lane, D. 1988. Isolation of protein antigens from bacterial overexpression systems: inclusion bodies. In *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory. pp. 726.

Harrison, B.D., Mayo, M.A., and Baulcombe, D.C. 1987. Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature 328:799-802.

Hiebert, E., and McDonald, J.G. 1976. Capsid protein heterogeneity in turnip mosaic virus. Virology 70:144-150.

Hollings, M., and Brunt, A.A. 1981. Potyviruses. In *Handbook of Plant Virus Infections and Comparative Diagnosis*, pp.732-807. Edited by E. Kurstak. Amsterdam, Elsevier.

Horsewood, P., McDermott, M.R., Stobbs, L.W., Brais, P.L.J., and Underdown, B.J. 1991. Characterization of a monoclonal antibody to TuMV and its use in immunodiagnosis of infection. Phytoprotection 72:61-68.

Horváth, J. 1979. New artificial hosts and non-hosts of plant viruses and their role in the identification and separation of viruses. IX. Potyvirus group (subdivision-III): Potato virus Y, turnip mosaic virus and watermelon mosaic virus (strain 2 or general strain). Acta Phytopath. Acad. Sci. Hung. 14:157-173.

Keen, N.T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. 24:447-463.

Kesseli, R.V., Paran, I., and Michelmore, R.W. 1991. Genetic linkage map of lettuce (*Lactuca sativa*, 2n=18). In *Genetic Maps*. S.J. O'Brien ed. Cold Spring Harbor Laboratory. 6:100-102.

Knorr, D.A., and Dawson, W.O. 1988. A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*. Proc. Natl. Acad. Sci. USA 85:170-174.

Kombrink, E., Schroder, M., Hahlbrock, K. 1988. Several "pathogenesis-related" proteins in potato are 1,2-B-glucanases and chitinases. Proc. Natl. Acad. Sci. USA 85:782-786.

Laliberté, J.-F., Nicolas, O., Chatel, H., Laure, C., and Morosoli, R. 1992. Release of a 22-kDa protein derived from the amino-terminal domain of the 49-kDa NIa

of turnip mosaic potyvirus in *Escherichia coli*. Virology 190:510-514.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Dally, M.J., Lincoln, S., and Newburg, L. 1987. Mapmaker: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181.

Landry, B.S., kesseli, R.V., Farrara, B., Michelmore, R.W. 1987. A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance, and morphological markers. Genetics 116:331-337.

Legrand, M., Kauffmann, S., Geoffroy, P, and Fritig, B. 1987. Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc. Natl. Acad. Sci. USA 84:6750-6754.

Loebenstein, G., and Gera, A. 1981. Inhibitor of virus replication released from tobacco mosaic virus-infected protoplasts of a local lesion-responding tobacco cultivar. Virology 114:132-139.

Meinkoth, J. and Wahl, G. 1984. Hybridization of nucleic acids immobilied on solid supports. Anal. Biochem. 138:267.

Martin, G.B., de Vicente, M.C., and Tanksley, S.D. 1993. High-resolution linkage analysis and physical characterization of the *Pto* bacterial resistance locus in tomato. Mol. Plant-Microbe Interactions 6:26-34.

Matthews, R.E.F. 1991. Factors influencing the course of infection and disease in *Plant Virology*, third edition. Toronto, Academic Press, Inc. pp. 455-457.

McDonald, J.G., and Hiebert, E. 1975. Characterization of the capsid and cylindrical inclusion proteins of three strains of turnip mosaic virus. Virology 63:295-303.

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N., and Okada, Y. 1988. Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene. EMBO J. 6:2557-2563.

Michelmore, R.W., Paran, I., and Kesseli, V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. U.S.A. 88:9828-9832.

Nicolas, O., and Laliberté, J.-F. 1991. The use of PCR for cloning of large cDNA fragments of turnip mosaic potyvirus. J. Virol. Methods. 32:57-66.

Nicolas, O. and Laliberté, J.-F. 1992. The complete nucleotide sequence of turnip mosaic potyvirus RNA. J. Gen. Virol. 73:2785-2793.

O'Brien,S.J. 1990. *Genetic Maps: Locus Maps of Complex Genomes.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Padgett, H.S., and Beachy, R.N. 1993. Analysis of a tobacco mosaic virus strain capable of overcoming *N* gene-mediated resistance. Plant Cell 5:577-586.

Paran, I., Kesseli, R., and Michelmore, R. 1991. Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near-isogenic lines. Genome 34:1021-1027.

Paran, I., and Michelmore, R.W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85:985-993.

Pierpoint, W.S. 1983. The major proteins in extracts of tobacco leaves that are responding hypersensitively to virus infection. Phytochemistry 22:2691-2697.

Pierpoint, W.S. 1986. The pathogenesis-related proteins of tobacco leaves. Phytochemistry 25:1595-1601.

Pink, D.A.C., and Walkey, D.G.A. 1990. Resistance to turnip mosaic virus in white cabbage. Euphytica 51:101-107.

Ponz, F., Glascock, C.B., and Bruening, B. 1988. An inhibitor of polyprotein processing with the characteristics of a natural virus resistance factor. Mol. Plant-Microbe Interact. 1:25-31.

Raptis, L. 1975. M.Sc. thesis. Macdonald campus of McGill University.

Redolfi, P. 1983. Occurrence of pathogenesis-related (b) proteins in different plant species. Neth. J. Plant Pathol. 89:245-254.

Reiter, R.S., Williams, J., Feldmann, K.A., Rafalski, J.A., Tingey, S.V., and Scolnick, P.A. 1992. Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. Proc. Natl. Acad. Sci. USA 89:1477-1481.

Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M.,

Riordan, J.R., Tsui, L., Collins, F.S. 1989. Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 245:1059-1065.

Ryder, E.J. 1983. Inheritance, linkage, and gene interaction studies in lettuce. J. Amer. Soc. Hort. Sci. 108:985-991.

Saito, T., Meshi, T., Takamatsu, N., and Okada, Y. 1987. Coat protein gene sequence of tobacco mosaic virus encodes a host response determinant. Proc. Natl. Acad. Sci. USA 84:6074-6077.

Saiki, R.K., Scarf, S., Faloona, F., Mulks, K.B., Horn, G.T., Erlich, H.A., Arnheim, N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anemia. Science 230:1350-1354.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Science 239:487-491.

Shattuck, V.I., and Stobbs, L.W. 1987. Evaluation of rutabaga cultivars for turnip mosaic virus resistance and inheritance of resistance. HortScience 22:935-937.

Shattuck, V.I., Brolley, B., Stobbs, S.W., and Lougheed, E.C. 1989. The effect of turnip mosaic virus infection on the mineral content and storability of field-grown rutabaga. Communications in Soil Science and Plant Analysis 20:581-595.

Singer, M., and Berg, P. 1991. Genes and Genomes. Edited by Carol Dempster. California: University Science Books. pp. 929.

Smith, K.M. 1972. *A Textbook of Plant Virus Diseases*, third edition. Longman Group Limited. pp. 558-560.

Spiegel, S., Gera, A., Salomon, R., Ahl, P., Harlap, S., and Loebenstein, G. 1989. Recovery of an inhibitor of virus replication from the intercellular fluid of hypersensitive tobacco infected with tobacco mosaic virus and from uninfected induced-resistant tissue. Phytopath. 79:258-262.

Stam, P., and Zeven, A.C. 1981. The theoretical proportion of the donor genome in near-isogenic lines of self-fertilizers bred by backcrossing. Euphytica 30:227-238.

Studier, F.M., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzym. 185:60-89.

Tanksley, S.D. 1983. Gene Mapping. In *Isoenzymes in Plant Genetics and Breeding*,

part A. Edited by S.D. Tanksley and T.J. Orton, pp. 111-140. Amsterdam: Elsevier.

Tanksley, S.D., Young, N.D., Paterson, A.H., and Bonierbale, M.W. 1989. RFLP mapping in plant breeding: New tools for an old science. Bio/technology 7:257-264.

Tanksley, S.D., and Mutschler, M.A. 1990. Linkage map of tomato (*Lycopersicon esculentum*). In *Genetic maps*, 5th edition, S.J. O'Brien (ed). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Tanksley, S.D., Ganal, M.W., Prince, J.P., de Vincente, M.C., Bonierbale, M.W., Broun, P., Fulton, T.M., Giovannoni, J.J., Grandillo, S., Martin, G.B., Messegeur, R., Miller, J.C., Miller, L., Paterson, A.H., pineda, O., Roder, M.S., Wing, R.A., Wu, W., and Young, N.D. 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141-1160.

Timmerman, G.M., Frew, T.J., Miller, A.L., Weeden, N.F., and Jermyn, W.A. 1993. Linkage mapping of *sbm-1*, a gene conferring resistance to pea seed-borne mosaic virus, using molecular markers in *Pisum sativum*. Theor. Appl. Genet. 85:609-615.

Tingey, S.V., Rafalski, J.A., and Williams, J.G.K. 1992. Genetic analysis with RAPD markers. Proc. Symposium on Applications of RAPD Technology to Plant Breeding, Nov. 1, Minneaplolis, Minnesota. Joint Plant Breeding Symposia Series. Crop Science Society of America/American Society for Hort. Sci./ American Genetic Assoc. pp. 3-8.

Tinker, N.A., Fortin, M.G., and Mather, D.E. 1993. Random amplified polymorphic DNA and pedigree relationships in spring barley. Theor. Appl. Genet. 85:976-984.

Tomlinson, J.A. 1970. Turnip mosaic virus. AAB/CMI Descriptions of Plant Viruses No.8, 4 pp.

Tomlinson, J.A., and Ward, C.M. 1978. The reactions of swede (*Brassica napus*) to infection by turnip mosaic virus. Ann. appl. Biol. 89:61-69.

Tomlinson, J.A., and Ward, C.M. 1982. Selection for immunity in swede (*Brassica napus*) to infection by turnip mosaic virus. Ann. Applied Biol. 101:43-50.

Tremblay, M.-F., Nicolas, O., Sinha, R.C., Lazure, C., and Laliberté, J.-F. 1990. Sequence of the 3'-terminal of turnip mosaic virus RNA and the capsid protein gene. J. Gen. Virol. 71:2769-2772.

Vaitukaitis, J.L. 1981. Production of antisera with small doses of immunogen: multiple intradermal injections. Meth. Enzym. 73:46-52.

van Loon, L.C. 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4:111-116.

van Loon, L.C. 1987. Disease induction by plant viruses. Adv. Virus Res. 33:205-255.

Wahl, G.M., Stern, M., and Stark, G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA 76:3683.

Walkey, D.G.A. 1985. Control through resistant cultivars. In *Applied Plant Virology*, Wiley-Interscience, New York. pp. 251-252.

Walkey, D.G.A., and Neely, H.A. 1980. Resistance in white cabbage to necrosis caused by turnip and cauliflower mosaic viruses and pepper-spot. J. Agric. Sci. 95:803-813.

Walkey, D.G.A., Ward, C.M., and Phelps, K. 1985. Studies on lettuce mosaic virus resistance in commercial lettuce cultivars. Plant Path. 34:545-551.

Ward, C.W., and Shukla, D.D. 1991. Taxonomy of potyviruses: current problems and some solutions. Intervirology 32:269-296.

Weeden, N.F., Timmerman, G.M., Hemmat, M., Kneen, B.E., and Lodhi, M.A. 1992. Inheritance and reliability of RAPD markers. Proc. Symp. Applications of RAPD Technology to Plant Breeding, Nov.1, Minneapolis, Minnesota. Joint Plant Breeding Symposia Series. Crop Sci. Soc. of Amer./Amer. Soc. for Hort. Sci./Amer. Genetic Assoc. pp. 12-17.

White, R.F. 1979. Acetyl salicylic acid (aspirin) induces resistance to TMV in tobacco. Virology 99:410.

White, R.F., and Antoniw, J.F. 1991. Virus-induced resistance responses in plants. Critical Rev. Plant Sci. 9:443-451.

Wicking, C., and Williamson, B. 1991. From linked marker to gene. Trends in Genetics 7:288-293.

Wilson, T.M.A. 1993. Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms. Proc. Natl. Acad. Sci. USA 90:3134-3141.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers.

Nucleic Acids Res. 18:6531-6535.

Young, N.D., Zamir, D., Ganal, M.W., and Tanksley, S.D. 1988. Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *Tm-2a* gene in tomato. Genetics 120:579-585.

Young, N.D., and Tanksley, S.D. 1989. RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of tomato during backcross breeding. Theor. Appl. Genet. 77:353-359.

Zabeau, M., and Roberts, R.J. 1979. The role of restriction endonucleases in molecular genetics. In *Molecular Genetics*, vol III, Taylor, J.H. (ed.). New York: Academic Press. pp. 1-63.

Zink, F.W., and Duffus, J.E. 1969. Relationship of turnip mosaic virus susceptibility and downy mildew (*Bremia lactucae*) resistance in lettuce. J. Amer. Soc. Hort. Sci. 94:403-407.

Zink, F.W., and Duffus, J.E. 1970. Linkage of turnip mosaic virus susceptibility and downy mildew, (*Bremia lactucae*), resistance in lettuce. J. Amer. Soc. Hort. Sci. 95:420-422.